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# OLOGY

AMERICAN

# THE RELATION OF VITAMINES TO THE GROWTH OF A STREPTOCOCCUS[1]

S. HENRY AYERS AND COURTLAND S. MUDGE

*From the Research Laboratories of the Dairy Division, United States Department of Agriculture*

The importance of vitamines in animal nutrition has led to a little experimental work with bacteria. Results of various investigators have caused the belief that certain materials such as animal and plant tissues contain growth accessory substances which stimulate the growth of microörganisms and which with some bacteria are essential for growth.

Among the studies along this line may be mentioned the work of Cole and Lloyd (1917), Paccinni and Russell (1918), Hall (1918), Agulhon and Legroux (1918), Kligler (1919), Bachman (1919), Williams (1919), Willaman (1920), Davis (1921), Rivers and Poole (1921), and MacLeod and Wyon (1921). In some papers the growth-promoting substances are spoken of as growth-accessories substances, and in others as vitamines. The work in general clearly indicates that for some microörganisms there are present in certain materials growth-promoting substances. As long as this term is used, one is not committed to the assumption that these substances may be vitamines. Glucose in small amounts is a growth-promoting substance for many bacteria, in fact any easily available source of carbon can be considered a growth-promoting substance. If, however, the substances which have been found to be growth promoters are classed as vitamines, then there is a great possibility of investigators being misled as to the real connection between the recognized vitamines and the growth of microörganisms.

In this paper we shall present the results of a few experiments

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with the water-soluble vitamine B and the fat-soluble vitamine A in their relation to the growth of a streptococcus.

## EXPERIMENTS WITH WATER-SOLUBLE B

Throughout our experiments we have used a culture of a pathogenic streptococcus which grew slowly in a peptone yeast medium. This organism would not grow in a peptone medium without broth, and the fact that it would grow with the addition of yeast extract brought up the possibility that the water-soluble B might be the reason for the growth.

Several experiments were conducted in order to throw some light on this point. In the first experiment 10 grams of autolized yeast were extracted in a Soxhlet apparatus for eight hours with 95 per cent alcohol. Fresh alcohol was then added and the extraction continued for another eight hours. This process was repeated until the extraction had been continued for forty hours. During the extraction the yeast was removed several times from the thimble and the clumps were thoroughly disintegrated. As the alcohol was removed during the extraction it was evaporated at a temperature of 60° to 80°C. The combined residues from the successive extractions were added to 500 cc. of distilled water and heated for a half hour. To this was added an equal amount of 2 per cent Difco peptone and the reaction adjusted to pH 7.2. This medium was then steamed for fifteen minutes, filtered, placed in flasks, and sterilized. The residue from the original 10 grams of yeast which had undergone extraction by the 95 per cent alcohol was added to 500 cc. of distilled water and another medium prepared with Difco peptone as described. A third medium was prepared by using 10 grams of the regular autolized yeast which had not been extracted. This was added to 500 cc. of distilled water and a medium prepared with Difco peptone, the same as with the other media. In all the experiments the media were distributed in 50 cc. amounts in 100-cc. Erlenmeyer flasks. These flasks were approximately of the same shape so that the depth of the medium and the surface area exposed was about the same in each flask.

The flasks containing each of the three media described were inoculated with a twenty-four hour culture of the streptococcus and incubated at 37°C. Examinations for growth were made after seventeen, twenty-four and forty-eight hours. Assuming that the water-soluble B can be extracted by hot 95 per cent alcohol, the medium made up with the alcohol extract should

TABLE 1

*Growth of a streptococcus in various media*

| MEDIA | HOURS OF INCUBATION | | | REMARKS |
|---|---|---|---|---|
| | 18 | 24 | 48 | |
| 95 per cent alcoholic yeast extract + peptone | − | − | +* | Should contain water-soluble B |
| Extract from yeast residue + peptone | +* | + | ++ | Should not contain water-soluble B |
| Extract of regular yeast + peptone | +* | + | ++ | Contains water-soluble B |
| Fraction I + peptone | − | − | − | Should contain some water-soluble B |
| Fraction II + peptone | − | − | − | Should contain most water-soluble B |
| Filtrate A + peptone | − | + | ++ | Should contain little if any water-soluble B |
| Yeast extract treated with Lloyd's Reagent + peptone | − | + | ++ | Should not contain water-soluble B from yeast |
| Regular yeast extract + peptone | − | + | ++ | Should contain water-soluble B from yeast |

* Slight.

contain this vitamine. The medium prepared from the alcohol-extracted yeast should not contain the water-soluble B, or at least the amount should be greatly reduced. Further, the medium made from the regular yeast should of course contain the vitamine.

The results shown in table 1 were of considerable interest to us. The growth was recorded by + signs which were increased

in number as the growth seemed heavier. It will be observed that the growth was equally good in the medium prepared from the regular yeast and from the yeast extracted with the alcohol, whereas only a slight growth was manifest after forty-eight hours in the medium containing the alcoholic extract of yeast. The results would seem to indicate that water-soluble B is not the growth-promoting substance of yeast at least for the streptococcus used in this work.

There is, of course, a possibility that the water-soluble B was not soluble in the 95 per cent alcohol and was not contained in the extract, and for this reason another experiment was conducted in which a method similar to that of Osborn and Wakeman (1919) was used for the preparation of fractions containing different amounts of water-soluble B. Some of the same autolized yeast was used, 10 grams being added to 500 cc. of boiling water containing 0.01 per cent acetic acid. This was stirred for some time and filtered, the filtrate being concentrated to 300 cc. at a temperature of about 80°C. To this 300 cc. of filtrate sufficient 95 per cent alcohol was added to get a concentration of about 52 per cent. The solution was allowed to stand over night at about 5°C. and the precipitate which had appeared the next day was filtered off. This precipitate was known as *fraction I*. The filtrate was then concentrated to 200 cc. and sufficient 95 per cent alcohol added to give a concentration of 79 per cent. This solution also was allowed to stand over night at a temperature of 5°C., and the precipitate filtered off. This was known as *fraction II*. The filtrate was again concentrated, this time to 100 cc., and sufficient 95 per cent alcohol added to give a concentration of about 90 per cent. After standing over night at 5°C. the precipitate that formed was filtered off and kept as *fraction III*. The filtrate from fraction III, which we shall term *filtrate A*, was evaporated to dryness, and dissolved in 1000 cc. of distilled water, to which 1 per cent of Difco peptone was added, the reaction adjusted to pH 7.2 and the medium placed in flasks and sterilized in an autoclave. Fractions I and II were each dissolved in 1000 cc. of distilled water and 1 per cent Difco peptone was added to each lot. These media were adjusted, placed in flasks

and sterilized as described above. Osborn and Wakeman found that fraction II contained most of the water-soluble B, and that fractions I and III contained some. According to this we should expect that the peptone medium containing fraction II should contain most of the water-soluble B, that the medium containing fraction I should contain less, and that filtrate A should contain the least or none at all. On this assumption, that water-soluble B is the growth-promoting substance of yeast, it would be expected that media containing fraction II would give the best growth with the streptococcus. From the second section of table I it will be seen that such was not the case. There was no growth in peptone media containing fraction II or fraction I, but there was growth in the peptone medium containing filtrate A. These results seem to confirm those obtained in the first experiment and again indicate that water-soluble B is not the growth-promoting substance of yeast.

A third experiment was conducted in which the water-soluble B was considerably reduced and possibly entirely removed from the yeast extract. Seidell (1916) has shown that, by shaking autolized yeast extract with Lloyd's Reagent (Fuller's Earth) using 50 grams per liter of extract, only an inconsiderable amount of the vitamine appeared to remain in the filtrate. Ten grams autolized yeast were added to 200 cc. of distilled water. This was allowed to stand for about one hour with frequent shaking before filtering. To this filtrate 50 grams of Lloyd's Reagent were added together with HCl to make the solution 0.01 normal. The yeast extract was then shaken every half hour for four hours and the Lloyd's Reagent removed by filtration. The Lloyd's Reagent was supplied to us through the kindness of Dr. Seidell and was known to be an active grade of Fuller's Earth. The filtrate was made up to 1000 cc. to which 1 per cent of Difco peptone was added and the reaction adjusted to pH 7.2. After filtration this medium was put in flasks and sterilized in the usual way in the autoclave. Since it has been found that the Lloyd's Reagent removes a large part, if not all, of the water-soluble B from yeast extract it would be expected that a peptone medium containing a yeast extract treated with this substance would not

support growth of the streptococcus. The results shown in the third section of table I indicate that the growth in such a medium was just as good as the growth in our regular peptone medium containing the extract of autolized yeast.

We have only shown in our tables the result of growth in a single flask, but these results have been duplicated many times so that it is felt that the results presented express the general average.

The results of MacLeod and Wyon (1921) are interesting in connection with our experiments. They conducted some experiments on the growth of a streptococcus with extracts from various substances. The used extracts from liver, kidney, egg yolk, yeast, muscle, bran, and milk, and found that with the exception of milk and bran the results seemed to favor the vitamine hypothesis so far as small amounts were active. They found however, that yeast extract had but little more growth promoting power than muscle extract. In view of the high water-soluble B content of yeast, and of its small amount or even absence in muscle, these results appear to us again to indicate that the water-soluble B is not the growth-promoting substance of yeast.

MacLeod and Wyon also worked with the pneumococcus and meningococcus and they point out that the growth-promoting property of certain extracts did not bear a direct relation to the known vitamine content, and that yeast had little or no effect in promoting the growth of these organisms.

A survey of the second and third experiments shows that an attempt was made in two distinct ways to obtain data on the relation of water-soluble B to the growth-promoting substance or substances of yeast. In the second experiment accepted methods were employed for removing a fraction from yeast extract containing a large amount of water-soluble B. This fraction with peptone did not support growth of the streptococcus and this fact is very significant. In the third experiment accepted means were used for removing or at least greatly reducing the water-soluble B content of the yeast extract. This extract which should have been free or very nearly free from water-soluble B, with peptone, supported growth of the streptococcus in

a normal manner. The results of these experiments when interpreted in light of our present knowledge of water-soluble B, and applied to the streptococcus studied, permit only the conclusion that this vitamine is not the growth-promoting substance of yeast extract. It is interesting to note that Funk and Dubin (1921) working on the vitamine requirements of yeasts and bacteria, have isolated a substance which they believe to be definite and specific for the stimulation of the growth of yeast. This substance, which was separated from vitamine B, they call vitamine D.

### EXPERIMENTS WITH CABBAGE EXTRACTS

It has been found that extracts from plant tissues apparently contain growth promoting substances for microörganisms, and cabbage seems to have given particularly good results. We tried one experiment in order to determine whether or not cabbage extract was valuable for the growth promotion of the streptococcus. One hundred cubic centimeters of finely minced cabbage were added to 300 cc. of distilled water and allowed to stand for forty-eight hours in the ice box. The suspension was then steamed for thirty minutes and filtered. Fifty cubic centimeters of 2 per cent Difco peptone solution were distributed into a series of flasks. To these flasks were added increasing amounts of the cabbage extract, these amounts being 1 cc., 5 cc., and 50 cc. Whenever necessary the medium was made up to 100 cc. and dispensed into tubes. Thus we had a series of media, one containing 1 per cent peptone alone and the other three having 1 per cent, 5 per cent, and 50 per cent of cabbage extract. Series of each of these media were inoculated with several different cultures (one of them being the culture used in the other experiments), were incubated for twenty-four hours, and then examined for growth and reaction. It was found that in the plain peptone without cabbage extract there was a fair growth of some of the streptococci, but with others the growth was questionable. In all of the tubes containing cabbage extract, growth was observed and the increase was more or less proportional to the increase in percentage of cabbage extract. The acidity also increased in a

similar manner. These results showed quite plainly that the cabbage extract contained some growth promoting substance, but the increase in acidity with the increase in percentage of cabbage extract also indicated that there was sugar present. This fact led us to consider the effect of sugar as a growth promoting substance. An analysis of the cabbage extract for which we are indebted to Doctor Rupp of these laboratories, showed that it contained 1.4 per cent of reducing sugar. Calculations showed that when the cabbage extract was incorporated with the peptone solutions the resulting media contained 0.014, 0.07 and 0.7 per cent of sugar for the 1, 5, and 50 per cent cabbage media, respectively. In order to show the effect of these percentages of sugar on the growth of the streptococcus a series of media was prepared as previously described except that a 1.4 per cent glucose solution was incorporated in the media instead of the cabbage extract. The growth in these last media was compared with that in the cabbage extract media mentioned.

From the results of this comparison, shown in table 2, it will be seen that growth in peptone media with cabbage extract was practically identical with that in the peptone media with the glucose solution. The growth increased, generally speaking, with the increase in the percentage of cabbage extract, and, in a similar manner, with the increase in the percentage of glucose solution. It should be remembered that the glucose solution contained 1.4 per cent reducing sugar so as to correspond with the cabbage extract. It is particularly interesting to note the increase in growth due to the incorporation of only 1 per cent of the glucose solution which gave a sugar content *in the medium* of only 0.014 per cent. From these results it seems evident that a very small amount of sugar acts as a growth-promoting substance for some of the streptococci.

We wish to emphasize that when extracts of plant tissues are used as a source of growth-promoting substances, the possible effect of sugar and other reducing substances present must be given considerable thought before the growth promotion can be attributed to vitamines. This is not only true when plant tissues are used but holds equally well for any extracts which may contain sugars.

TABLE 2

*Effect on growth of streptococci of addition of cabbage extract and glucose to a peptone medium*

| CULTURE NUMBER | NONE | | | | 1 PER CENT | | | | 5 PER CENT | | | | 50 PER CENT | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Cabbage extract | | Glucose solution | | Cabbage extract | | Glucose solution | | Cabbage extract | | Glucose solution | | Cabbage extract | | Glucose solution | |
| | Growth | pH | Growth | pH | Growth | pH | Growth | pH | Growth | pH | Growth | pH | Growth | pH | Growth | pH |
| 1 | + (?) | 7.6 | + (?) | 7.6 | ++ | 6.8 | ++ | 7.2 | ++++ | 6.2 | +++ | 5.4 | ++++ | 6.0 | +++ | 5.4 |
| 2 | + | 7.6 | + | 7.6 | ++ | 7.0 | ++ | 7.2 | ++++ | 5.6 | +++ | 5.4 | ++++ | 5.4 | +++ | 5.4 |
| 3 | + | 7.8 | + | 7.6 | ++ | 6.9 | ++ | 7.2 | ++++ | 4.8 | +++ | 5.4 | ++++ | 4.5 | ++++ | 4.5 |
| 4 | + | 7.4 | + | 7.4 | ++ | 6.8 | ++ | 7.0 | ++++ | 4.5 | ++++ | 5.0 | ++++ | 4.5 | ++++ | 4.5 |
| 5 | + | 7.8 | + | 7.8 | ++ | 6.9 | ++ | 7.4 | ++++ | 5.4 | ++++ | 5.5 | ++++ | 4.5 | ++++ | 4.5 |

## EXPERIMENTS WITH FATS AND OILS

We became interested in the effect of fats and oils as sources of growth promoting substances from the results obtained by Dr. Sherman of these laboratories, who found that fat-soluble A was apparently necessary for the growth of some of the organisms of the high acid group.

In all of our experiments the following basic medium was used:

| | |
|---|---|
| Peptone (Difco) | 10 grams |
| Autolized yeast | 10 grams |
| Distilled water | 1,000 cc. |
| pH | 7.4 |

Fifty cubic centimeters of this medium were placed in 100 cc. Erlenmeyer flasks and 0.5 cc. of the fat or oil to be studied was added before sterilization at 15 pounds for thirty minutes. This gave a concentration of 1 per cent of the oil in the medium. In the first experiments butter-fat and cod-liver oil were used. The butterfat was prepared from sweet cream churned into butter and washed with warm water several times. As a final process the fat was filtered through paper into a clean, dry beaker in which it was stored at about 5°C. during the course of these experiments. Using the same culture of streptococcus employed in the experiments with water-soluble B it was found that both butterfat and cod-liver oil stimulated the growth considerably during the first twenty-four hours. These experiments were repeated several times and the same results were obtained. While these results indicated that fat-soluble A might be responsible for the stimulation it was decided to try olive oil which is believed not to contain this vitamine. The same stimulation occurred however as with butterfat and cod-liver oil. This seemed to present evidence that fat-soluble A was not responsible for the stimulation.

While it is generally considered that olive oil does not contain fat-soluble A, the work of Drummond and Coward (1920) indicates that it may be present in small amounts. For this reason it was decided to use oil which could hardly be suspected of containing fat-soluble A and white mineral oil was therefore

selected. Much to our surprise the same stimulation of growth of the streptococcus occurred as with butterfat and cod-liver oil.

In these prelininary experiments there was no accurate measure of the degree of stimulation of growth, for it had been judged only by the appearance of the turbidity of the cultures after shaking. A more accurate measure was desired and the following method was adopted. Flasks containing 50 cc. of the yeast peptone medium with 1 per cent of a number of different oils[2] were inoculated with a loopful of a water suspension of a twenty-four-hour growth of the streptococcus on agar. After twenty-four hours' incubation at 37°C. the amount of growth was determined by plating on infusion agar. The results of these experiments are shown in table 3. It will be seen that the inoculated control peptone yeast medium without oil contained 170,000 bacteria per cubic centimeter after twenty-four hours of incubation. With sesame oil there was apparently little if any stimulation, while chaulmoogra oil seemed to be toxic. The rest of the oils and fats increased the count to a considerable degree. Some of the vegetable oils were considerably active, as for example okra-seed oil. This result is based on only one experiment, and in a repetition of the experiment a count was obtained with okra-seed oil similar to those observed with the other oils.

The most interesting feature of the results was the marked stimulation of growth by mineral oil, vaseline, and even solid paraffin. Microscopic examinations were made in some cases to prove that the plate count represented a difference in the actual number of cells in the cultures. Since we were working with a chain-forming streptococcus, the influence of the oil might have been to decrease chain formation, and therefore apparently increase the count. Microscopic tests showed, however, that the plate count represented the real difference in growth.

The results so far were obtained in media containing 1 per cent of the various oils but it was thought that this percentage could be lowered. To this end oils were selected from the three

[2] We are indebted to Doctor Jameison of the Bureau of Chemistry for many of the oils used in our experiments.

major groups (animal, vegetable, and mineral) and the amount of fat diminished to 0.2 per cent, 0.1 per cent, 0.02 per cent and 0.002 per cent. In table 4 the results are given. It will be seen that the counts tended to decrease with the lessened amount

TABLE 3

*Effect of various fats and oils on the growth of a streptococcus*

| FAT OR OIL (1 PER CENT USED) | REMARKS | BACTERIA PER CUBIC CENTIMETER AFTER TWENTY-FOUR HOURS |
|---|---|---|
| Control without oil | | 170,000 |
| Sesame | | 400,000 |
| Chaulmoogra | | less than 100,000 |
| Castor | | 1,100,000 |
| Corn | Crude | 2,600,000 |
| Rape seed | Crude | 3,500,000 |
| Lumbang | Candle nuts | 4,100,000 |
| Coconut | Refined | 6,000,000 |
| Peanut | Crude | 8,800,000 |
| Peanut | Refined | 11,000,000 |
| Soy bean | | 10,400,000 |
| Olive | | 9,700,000 |
| Chia seed | | 10,400,000 |
| Mustard seed | | 14,000,000 |
| Okra seed | | 33,000,000 |
| Linseed | Raw | 5,600,000 |
| Lard | Prime steamed | 3,900,000 |
| Lard | Open kettle rendered, direct heat | 1,300,000 |
| Lard | Prime steamed, refined | 5,100,000 |
| Butterfat | From sweet cream | 14,100,000 |
| Cod liver | | 8,100,000 |
| Mineral | 1 per cent added | 5,700,000 |
| Mineral | 2 per cent added | 5,100,000 |
| Vaseline | 1 per cent added | 9,100,000 |
| Vaseline | 2 per cent added | 9,700,000 |
| Paraffine, solid | 1 per cent added | 5,900,000 |
| Paraffine, solid | 2 per cent added | 11,100,000 |

of fat or oil but even small quantities caused a marked stimulation. In plating these oil media it was difficult to keep the 1 cc. sample drawn from the flask free from drops of oil and perhaps this is the explanation of some of the inconsistencies seen

in the tables. In making the dilutions of 0.02 per cent and 0.002 per cent of the fats and oils the following methods were used: To dilution blanks containing 100 cc. of 1 per cent peptone, 1 cc.of the oil was added and an emulsion obtained by thorough shaking. By adding 1 cc. and 0.1 cc. to our media we obtained the percentages of fat mentioned above. The main point to be observed however is that even down to 0.002 per cent of oil in the medium there was a stimulation of growth. This small

TABLE 4

*Effect of small amounts of butterfat, rape and mineral oils on the growth of a streptococcus*

| CONCENTRATION | BUTTERFAT | RAPE OIL | MINERAL OIL |
|---|---|---|---|
| | *Bacteria per cc.* | *Bacteria per cc.* | *Bacteria per cc.* |
| Control without fat or oil | 680,000 | 680,000 | 680,000 |
| 1.0 | 10,000,000 | 13,600,000 | 32,000,000 |
| 0.2 | 2,140,000 | 12,000,000 | 25,000,000 |
| 0.1 | 1,260,000 | 17,900,000 | 17,900,000 |
| 0.02 | 6,900,000 | 13,900,000 | 6,900,000 |
| 0.002 | 5,000,000 | 5,600,000 | 3,100,000 |
| Extracts (?) from fats and oils | 10,300,000 | 5,600,000 | 3,600,000 |

| | | *Bacteria per cc.* |
|---|---|---|
| Butter 1 per cent | first experiment | 430,000 |
| | second experiment | 0 in 1:10,000 dilution |
| Butterfat 1 per cent | first experiment | 11,000,000 |
| | second experiment | 14,000,000 |

amount of oil could hardly be distinguished on the surface of the media even on careful examination. At the same time other media were made in which the oils butterfat, rape and mineral or extracts from them, were added, in the following way: 100 cc. of water was shaken up with 5 cc. of the fat and oils for a period of three hours. The bottles were than set aside in the laboratory for several days after which time was a layer of oil at the surface of the water but the water itself was slightly cloudy. Two cubic centimeters of this emulsion, if it was an emulsion, since we dealt with pure oil and distilled water, were added to

the basic media and inoculated with the streptococcus. In table 4 experiment 3 we see a most astonishing stimulation of growth.

Another interesting phenomenon was observed which might well be mentioned at this point. In 1918, Mr. Johnson of these laboratories prepared in the field, some pasteurized-cream butter without salt and also separated some of the butterfat free from casein. These were sealed in sanitary cans and sent to Washington. Since then they have been in our incubators at temperatures averaging 30°C. These cans were opened recently and the fat found to be in a melted condition. Samples were taken of the supernatant fat from a butter can and also some of the oil from the can of butterfat free from casein. Media were made with these two fats using 1 per cent. The results which are also shown in table 4 are peculiar to say the least. The fat from the butter itself seems to be toxic but the stored fat free from casein stimulated bacterial growth the same as our fresh butterfat. One other point of interest is that the stimulating effects of fats and oils was not manifest to any degree in a plain peptone medium but was evident in the yeast peptone medium.

It was found by the addition of methylene blue that an anerobic condition did not exist in the media with fats and oils. It cannot be assumed therefore that an anaerobic condition was responsible for the stimulation of the growth of the streptococcus.

The experiments with fats and oils show one thing definitely which is that very small amounts of these materials stimulate in a most remarkable manner the growth of a streptococcus growing in a yeast peptone medium. If our studies had been limited to butterfat and cod-liver oil strong evidence could have been presented as to the stimulation by fat-soluble A. But the stimulating effect of mineral oils and even solid paraffin change the aspect of the situation.

Our results as we see them permit of one of three possibilities, first, that the growth-promoting property of fats and oils is not due to the fat-soluble A, or second, that if fat-soluble A is responsible then it must be contained in mineral oil. The second

possibility suggests that it may be desirable to study further the vitamine content of mineral oil. Third, that the stimulation with fats and oils containing vitamine-A and that with mineral oils is not due to the same thing. Facts in our possession at present do not suggest which possibility is the correct one.

## SUMMARY AND CONCLUSIONS

1. The results presented in this paper apply only to the growth of a culture of a pathogenic streptococcus and do not necessarily apply to bacteria in general.

2. Autolized yeast extract contained a growth-promoting substance or substances for the streptococcus studied. Water-soluble B did not, however, appear to be the significant substance.

3. Cabbage extract was found to promote growth, but a glucose solution containing the same amount of sugar as the cabbage extract showed a similar growth-promoting effect. It is evident that when extracts of plant or animal tissues are used the sugar content must be given consideration in connection with their growth-promoting properties.

4. Fats and oils, vegetable, animal and mineral even in very small amounts were found to stimulate the growth of the streptococcus. Either the growth-promoting property of fats and oils is not due to fat-soluble A, or this vitamine is present in mineral oils, or the stimulation is due to different causes in the case of the vitamine-containing fats and oils and the mineral oils.

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# SALT EFFECTS IN BACTERIAL GROWTH[1]

## II. THE GROWTH OF BACT. COLI IN RELATION TO H-ION CONCENTRATION

JAMES M. SHERMAN AND GEORGE E. HOLM

*From the Research Laboratories of the Dairy Division, United States Department of Agriculture, Washington, D. C.*

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It has been shown in a previous publication (Holm and Sherman, 1921) that sodium chloride and various other neutral salts in 0.20M concentration affected the rate of growth of Bact. coli. Using neutral salts with a common cation (sodium) but with various anions a marked difference was observed between the action of the various salts. The effect of the chlorides of sodium, potassium and ammonium seemed to be approximately the same while the calcium and iron salts tested retarded greatly or inhibited growth. These experiments were carried out at a pH of approximately 7.0 and with a salt concentration of 0.20M in 1 per cent pepton.

In as much as we know that there are limiting pH values for bacterial growth, varying with different organisms, it would be of interest and value to know just to what extent this neutral salt action is affected by various H-ion concentrations In the following experiments, as in our former communication, the rate of growth was determined by the time that expired between inoculation and the first sign of turbidity. The medium used was 1 per cent pepton to which had been added various amounts of salts, and the H-ion concentration adjusted by the use of concentrated HCl and NaOH solutions.

The effects of various concentrations of NaCl at various H-ion concentrations were first tried. The H-ion and salt concentra-

[1] Published with the permission of the Secretary of Agriculture.

tions, and the time for growth in each series, are shown in table 1. This table shows a decided accelerating effect upon growth with added NaCl in low concentrations. Although there was little difference between the effects of 0.10, 0.20 and 0.30M NaCl media upon growth, there seems to be an optimum effect at about 0.20M. The optimum H-ion concentration for growth either in controls or in pepton containing NaCl at various concentrations seems to be about the same, approximately pH 7.8. At optimum salt concentrations there is very little difference in the rate of growth over a wide range of H-ion concentration,

TABLE 1

*The rate of growth of Bact. coli in various concentrations of NaCl in 1 per cent peptone and at various H-ion concentrations*

| NaCl concentration | Series I | | Series II | | Series III | | Series IV | | Series V | |
|---|---|---|---|---|---|---|---|---|---|---|
| | pH | Hours | pH | Hours | pH | Hours | pH | Hours | pH | Hours |
| Control | 5.3 | 36 | 6.3 | 10¾ | 7.0 | 7 | 7.7 | 6 | 8.3 | 7 |
| 0.05M | 5.3 | 6¾ | 6.3 | 8½ | 7.1 | 5¾ | 7.7 | 3¾ | 8.3 | 4 |
| 0.10M | 5.3 | 4 | 6.4 | 4 | 7.1 | 4 | 7.8 | 3½ | 8.3 | 3½ |
| 0.20M | 5.3 | 4 | 6.5 | 3½ | 7.2 | 3½ | 7.8 | 3½ | 8.3 | 3½ |
| 0.30M | 5.3 | 4½ | 6.5 | 3¾ | 7.3 | 3¾ | 7.9 | 3¾ | 8.3 | 3½ |
| 0.40M | 5.3 | 5¼ | 6.5 | 4½ | 7.3 | 4 | 7.9 | 4 | 8.3 | 4 |

varying from 5.3 to 8.3 on the pH scale, while in the pepton solution alone the range is somewhat narrower. Beyond the range for optimum growth there seems to be a decided retardation for each small change of H-ion concentration. These results are brought out more clearly in figure 1 which shows a pronounced widening of the limiting pH values for growth with added NaCl, especially in optimum salt concentrations, and a retardation of growth for each small change in pH near the limiting values.

In order to ascertain if there was actually a shifting of the limits of growth, or merely a widening of the optimum range for growth, the effect of NaCl was tried at pH values representing the approximate limit of growth in the acid region. At a pH of 4.8 it was found that only rarely would Bact. coli grow in 1 per cent pepton at 37°C., but that it did grow quite readily in the

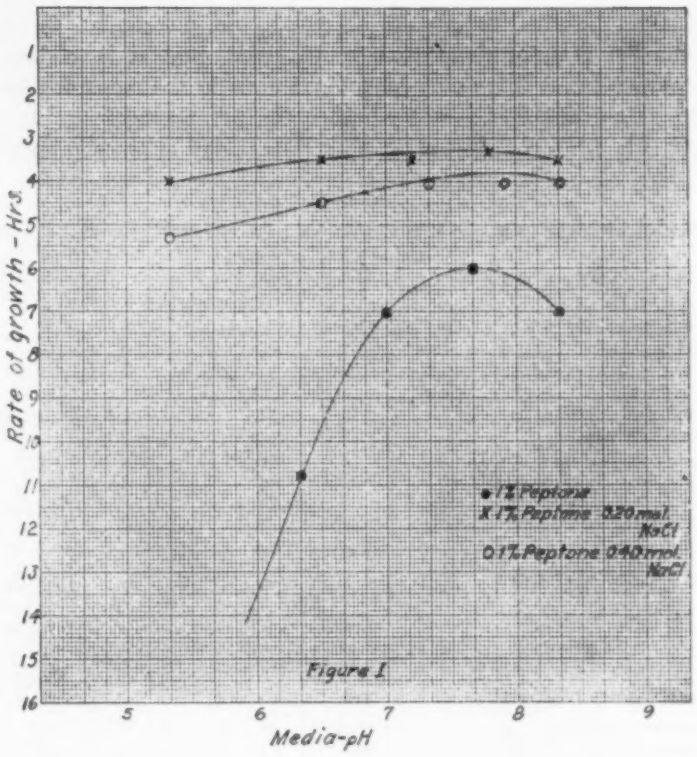



TABLE 2

*The shifting of the limit of growth of Bact. coli at 37°C. in the acid region by the addition of NaCl*

| TEST NUMBER | TIME REQUIRED TO SHOW TURBIDITY AT A pH VALUE OF 4.8 IN | |
|---|---|---|
| | 1 per cent pepton | 1 per cent pepton 0.20 M NaCl |
| | *hours* | *hours* |
| 1 | No growth | 18 |
| 2 | No growth | 22 |
| 3 | No growth | 18 |
| 4 | No growth | 18 |
| 5 | 26 | 21 |

same medium to which had been added NaCl to make a 0.20M solution. These media were adjusted colorimetrically, the end point being determined with methyl red, and it is of course recognized that there may be a slight error in the measurements. The significant fact is not whether the point established is exactly pH 4.8 but that the limiting H-ion zone of growth may be

TABLE 3

*The shifting of the limit of growth of Bact. alkaligenes at 37°C. in the acid region by the addition of NaCl*

| MEDIUM | TEST NUMBER | TIME REQUIRED TO SHOW TURBIDITY AT pH VALUES OF | | |
|---|---|---|---|---|
| | | 5.6 | 5.4 | 5.2 |
| | | hours | hours | hours |
| 1 per cent pepton | 1 | 90 | No growth | No growth |
| 1 per cent pepton | 2 | No growth | No growth | |
| 1 per cent pepton 0.20M NaCl | 1 | 24 | 24 | 120 |
| 1 per cent pepton 0.20M NaCl | 2 | 24 | 24 | |

TABLE 4

*The effect of NaCl and Na citrate upon Bact. coli at various H-ion concentrations*

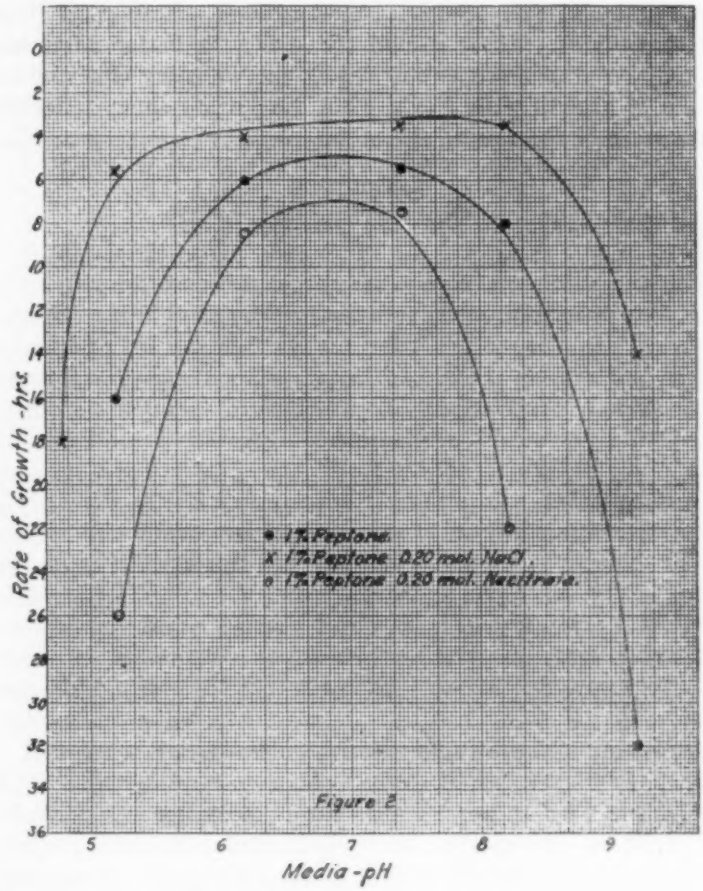
| MEDIA | TIME REQUIRED TO SHOW TURBIDITY AT pH VALUE OF | | | | | |
|---|---|---|---|---|---|---|
| | 4.8 | 5.2 | 6.2 | 7.6 | 8.2 | 9.2 |
| | hours | hours | hours | hours | hours | hours |
| 1 per cent pepton | No growth | 16 | 6 | 5½ | 8 | 32 |
| 1 per cent pepton 0.20M NaCl | 18 | 5½ | 4 | 3½ | 3½ | 14 |
| 1 per cent pepton 0.20M Na citrate | No growth | 26 | 8½ | 7½ | 22 | No growth |

modified by the addition of NaCl to the medium. The results of five different tests made under these conditions are recorded in table 2.

Although the widening effect upon the pH limit of growth is not general for all bacteria which we have tried, we have found the effect upon Bact. alkaligenes to be even more pronounced than the effect upon Bact. coli. This is shown in table 3.

Tables 2 and 3 show that there is actually an extension of the zone in which Bact. coli and Bact. alkaligenes will grow in the

acid region by the addition of 0.20M NaCl. It is possible that this effect might be increased by using a more dilute salt solution, and perhaps, in the same way, this effect might be produced




with organisms which thus far have failed to show any such modification in their limits of growth.

The same results which we have noted with NaCl may be produced with other neutral salts, but the degree of the widening

effect varies with the nature of the salt. On the other hand, a salt (e.g., Na citrate) which lowers the rate of growth also narrows the limits of H-ion concentration at which Bact. coli will grow. Table 4 shows the results obtained with NaCl and Na citrate, as compared with pepton alone, at different H-ion concentrations. These results are shown to better advantage in figure 2 which brings out clearly the widening effect on the limits of growth with NaCl, while Na citrate shows a decided narrowing of the range.

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# A NOTE ON THE MORPHOLOGY OF BACTERIA SYMBIOTIC IN THE TISSUES OF HIGHER ORGANISMS

IVAN E. WALLIN

*Department of Anatomy and the Henry S. Denison Research Laboratories, University of Colorado, Boulder*

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In connection with a study of the cytoplasmic relationships of root-nodule bacteria in the common white clover, the author found a stage in the morphogenesis of Bacillus radicicola that, apparently, is not well known. A careful search was made in textbooks of bacteriology for a description or mention of this form, but in vain. Through the courtesy of Dr. F. Löhnis of the United States Department of Agriculture, I have been able to find references to similar forms. Doctor Löhnis (1921) in his exhaustive review of the literature on the life cycles of the bacteria describes and illustrates round forms of Bacillus radicicola which appear to be similar to the spherical forms that I found. Löhnis and Smith (1916) were the first investigators to observe the spherical forms. More recently Bewley and Hutchinson (1920) have observed, apparently, the same form in cultural conditions and call this stage in the morphogenesis of the organism the "swarmer" stage.

In relation to the spherical forms that have been described by Löhnis (gonidia, regenerative units) it remains to be decided whether the spherical forms that I have called "senile" are derived from the branched forms (bacteroids) or from the symplasm. The relationship and character of these spherical forms in the root nodule is decidedly interesting and I believe that a study of these forms in sections of the root nodule may be valuable in the interpretation of their nature. It appears to the author that these spherical forms are fragile and are generally destroyed in the ordinary bacteriological technique.

When my specimens were first examined with a low magnification of the microscope I was impressed by what appeared to be three distinct regions or areas in the root nodule. On closer examination with oil immersion lens, the three areas were found to contain three distinct forms of organisms. Each form was more or less limited to a single area. In the part of the nodule next to the plant root, the nodule cells contained no other than the spherical forms. I interpret this part of the nodule to be the older part and on the basis of this interpretation have called the spherical cells "senile" forms. The "bacteroid" forms of the bacillus were likewise limited, almost entirely, to the central portion of the nodule. In what I interpret to be the younger part of the nodule the bacilli were all of the small variety and not nearly so numerous as the other forms.

My interest in these forms is not "bacteriological" and I have no desire to pursue the investigation any further, at least for the present. However, it does occur to me that the technique that I have used may be valuable in bacteriological research. In a recent publication (Wallin, 1922a), I submitted evidence that mitochondrial methods are not specific, but will also stain bacteria. It has since occurred to me, particularly in connection with my study of root-nodule bacteria, that the mitochondrial technique may, at least in some cases, be superior to the usual bacteriological methods, particularly when dealing with symbiotic bacteria. I have tested a number of mitochondrial methods on various kinds of bacteriological material: sputum smears, pus smears and sections, tissue smears, bacterial smears, etc. In the majority of instances the differentiation between bacterium and tissue has been decidedly sharp.

In staining the root nodules, I have used only one method. This consisted in fixation of the entire nodule in a modification of Flemming's fixative: 4 cc. 2 per cent aqueous solution of osmic acid and 6 cc. 1 per cent aqueous solution of chromic acid. (Fix from four to twenty-four hours.) After washing, dehydration, clearing, and embedding in hard paraffin (58°), sections were cut 3 micra in thickness. The sections were mounted on slides and stained by Bensley's (1911) anilin fuchsin-methyl green method:

The staining solutions are:

1. Altmann's acid fuchsin anilin solution:
   Acid fuchsin.....20 grams
   Anilin water.....100 cc.
2. 1 per cent solution of methyl green

The sections after being prepared for the staining process by treatment with permanganate of potassium followed by oxalic acid, are stained for five minutes in the acid fuchsin solution which has been previously warmed to 60°C. Next they are thoroughly washed in distilled water, and dipped for an instant into the solution of methyl green, then washed, rapidly dehydrated in absolute alcohol (alcohol of intermediate strength must be avoided) cleared in toluol, and mounted in balsam.

The author has found that the permanganate and oxalic acid treatment may be omitted if the fixation has not been carried too far. However, if the staining differentiation is not sharp the sections may be treated for a minute or so in 1 per cent permanganate of potassium and followed by a similar treatment in 5 per cent oxalic acid. I have also found a great variation in the quality of various brands of methyl green. I have only been able to get good results with Grübler's methyl green. This method, when carried out successfully, gives excellent differentiation between bacteria and tissues.

There are several mitochondrial methods in use. Benda's crystal violet method, perhaps, gives the sharpest differentiation of all these methods. This is a very long and tedious method, but in some cases the results appear to justify the longer procedure. Other methods are: Altmann's anilin fuchsin picric acid method, Schridde's modification of Altmann's method, Regaud's method, the copper and iron hemotoxylin methods, etc. I have stained bacteria by all these methods with good results.

The mitochondrial staining methods were devised to fix and stain delicate bodies (mitochondria) in the cytoplasm which are not visible after ordinary histological technique. It appears that some symbiotic bacteria, particularly those that have an intracellular relationship, are just as fragile and delicate as mito-

chondria. The author believes that his results on the root-nodule bacteria were due to this technique. For illustrations of the three forms of Bacillus radicicola, the reader is referred to an article by the author (Wallin, 1922b).

Murray (1919) has recently recommended the use of mitochondrial technique in certain cases where the Gram stain, for example, does not give differential results.

The author also wishes to call attention to the action of janus green when applied to bacteria. All bacteria, apparently, are not stained by janus green, but certain strains are and, apparently, only under certain conditions. This is a vital stain and it is possible that it may be found useful in the study of certain bacteria. The reader is referred to Cowdry (1918) for directions and information regarding the proper brand of janus green.

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# OBSERVATIONS ON THE PROPERTIES OF BACTERIOLYSANTS (D'HERELLE'S PHENOMENON, BACTERIOPHAGE, BACTERIOLYTIC AGENT, ETC.)

## PART I[1]

WILBURT C. DAVISON
*Baltimore, Maryland*

### INTRODUCTION

Twort, d'Herelle and others (Davison, 1922a) have observed that Berkfeld filtrates of stool and other cultures would kill and dissolve young cultures of dysentery and other organisms. The addition of a portion of one of these dissolved cultures, to a new culture would cause it in turn to dissolve. Thus the lytic principle could be transferred from generation to generation. These bacteriolytic filtrates or their subsequent generations are called, interchangeably, bacteriolysants, bacteriophages and bacteriolytic agents. The process by which these filtrates dissolve dysentery and other organisms is known as d'Herelle's phenomenon or bacteriophagy.

It has been shown (Davison, 1922a) that agar subcultures of organisms which had been attacked by bacteriophages, concontained two types of colonies. One was regular and round, resembling a typical dysentery colony. It was not readily attacked by the bacteriolytic agent. This is the "resistant" type. The other was irregular in outline and is best described as "moth eaten." These colonies were easily dissolved by bacteriophages and in addition had the property of dissolving other cultures to which they were added. This is the "sensitive"

[1] Presented at the twenty-third annual meeting, Society of American Bacteriologists, December 29, 1921. From the Department of Pediatrics, of the Johns Hopkins University and the Harriet Lane Home of the Johns Hopkins Hospital, Baltimore, Maryland.

or "lysogenic" strain. A "normal" strain is one that has never been in contact with a bacteriophage.

### STOOL CULTURE FILTRATES

I have obtained stool culture filtrates by inoculating a loopful of feces into 100 cc. of 1 per cent peptone water at pH 8. After incubation at 37°C. from twelve hours to five days, these cultures were filtered through a Mandler no. 6 candle. The filtrates were then incubated at 37°C. for forty-eight hours to prove their sterility. Filtrates were made from the stools of ten infants (table 1). Seven of these children suffered from bacillary dysentery (Flexner).[2] Two of these had Bact. dysenteriae in their stools at the time the filtrates were obtained. One of these two patients died. Two infants suffered from acute intestinal indigestion and one was a case of Otitis Media and feeding regulation. A bacteriophage obtained from the stool of an adult convalescent typhoid patient[3] has been used as a comparison. It was active against several normal strains of Flexner bacilli (table 1).

### METHODS OF TESTING THE BACTERIOLYTIC ACTIVITY OF BACTERIOLYSANTS

The bacteriolytic activity of these various filtrates was tested as follows: six tubes each containing 2 cc. of sterile 1 per cent peptone water (pH 8.0) were each inoculated with one drop of a fluid culture of Bact. dysenteriae (Flexner). After two to twelve hours' incubation at 37°C., 0.5 cc. of the filtrate to be tested was added to the first tube (making a dilution of 1:5), 0.25 cc. to the second tube (a dilution of 1:9), and 0.1 cc. to the third tube (a dilution of 1:21). Sterile peptone water in amounts of 0.5 cc., 0.25 cc. and 0.1 cc. was added to the fourth, fifth and sixth tubes which served as controls. These six tubes were

[2] By Flexner bacilli, I refer to the whole group of mannitol fermenting dysentery bacilli. By Flexner V, W, X, Y or Z, I refer to the English serological divisions of this group (Davison, 1922).

[3] This bacteriophage was given me by Miss Ann Kuttner of the Department of Bacteriology, Columbia University, to whom my thanks are due.

TABLE 1

*Bacteriolytic activity of stool culture filtrates*

| SOURCE OF BACTERIOLYSANT | | | NUMBER OF DIFFERENT CULTURES WITH WHICH BACTERIOLYSANTS WERE TESTED | NUMBER OF LYSIS TESTS MADE WITH ORIGINAL STOOL FILTRATE OR WITH SUBSEQUENT GENERATIONS OF IT | NUMBER OF NEGATIVE LYSIS TESTS | NUMBER OF SUBSEQUENT GENERATIONS TO WHICH ORIGINAL STOOL FILTRATE WAS CARRIED | LYTIC ACTIVITY OF SUBSEQUENT GENERATIONS COMPARED WITH THAT OF ORIGINAL STOOL FILTRATE | ACTIVITY OF BACTERIOLYSANT OBTAINED FROM PATIENT, AGAINST DYSENTERY BACILLUS ISOLATED FROM PATIENT AS COMPARED TO ITS ACTIVITY AGAINST OTHER STRAINS OF FLEXNER BACILLI |
|---|---|---|---|---|---|---|---|---|
| Name of patient | Diagnosis of patient | Outcome of case | | | | | | |
| J. B. (infant).... | Dysentery (Flexner) | Recovery | 9 | 17 | 3 | 6 | Same | Greater |
| E. B. (infant) ... | Dysentery (Flexner) | Recovery | 11 | 19 | 3 | 3 | Greater | Same |
| T. B. (infant) ... | Dysentery (Flexner) | Recovery | 16 | 31 | 2 | 6 | Same | Not tested |
| M. C. (infant)... | Dysentery (Flexner) | Recovery | 11 | 11 | 2 | 1 | Not tested | Not tested |
| I. J. (infant)..... | Dysentery (Flexner) | Recovery | 8 | 9 | 2 | 2 | Less | Less |
| D. R. (infant)... | Dysentery (Flexner) | Recovery | 6 | 11 | 0 | 4 | Same | Not tested |
| J. S. (infant).... | Dysentery (Flexner) | Death | 5 | 10 | 1 | 3 | Same | Less |
| A. L. (infant) ... | Acute intestinal indigestion | Recovery | 9 | 12 | 0 | 5 | Greater | |
| E. A. (infant) ... | Acute intestinal indigestion | Recovery | 1 | 1 | 0 | 0 | | |
| T. T. (infant) ... | Otitis media and regulation of feeding | Recovery | 20 | 75 | 4 | 10 | Greater | |
| Adult ..... | Typhoid fever | ? | 10 | 29 | 8 | 5 | Same | Not tested |

than incubated at 37°C. and the degree of lysis was noted macroscopically at various intervals for seventy-two hours.

Inasmuch as it had been shown (Davison, 1922a) that agar subcultures of organisms which have been attacked by bacteriolysants contain "sensitive," "moth eaten" colonies, the presence of such colonies in subcultures was used as confirmatory evidence of the occurrence of lysis. However, subcultures of some bacterial suspensions which had apparently been lysed by a filtrate contained nothing but regular colonies, so that their absence does not necessarily mean that lysis had not occurred.

Frequently when normal dysentery bacilli, which had been incubated with a bacteriolysant, were subcultured in peptone water these subcultures failed to grow, although agar subcultures made at the same time, contained a number of "sensitive" and regular colonies. It is possible that there was sufficient bacteriolysant carried over in the subculture loop to inhibit growth in a fluid medium while on agar, this small amount was lost in streaking across the plate.

The amount of bacteriolysant added to a culture of dysentery bacilli, apparently does not have a quantitative effect on the percentage of "sensitive" colonies which are found in agar subcultures of these bacilli, i.e., subcultures of peptone water cultures of dysentery bacilli some of which had been incubated with 0.5 cc. and others with 0.1 cc. of a bacteriolysant frequently had the same percentage of "sensitive" colonies.

In several instances I have confirmed these determination of lysis by counting the organisms (plate counts) before and after the action of a bacteriolysant and have found the reduction of viable organisms to be proportional to the degree of lysis, i.e., a culture which contained 100,000,000 Flexner bacilli per cubic centimeter at the commencement of the experiment, and which after twenty-four hours' contact with a filtrate was apparently completely lysed, contained two viable organisms per cubic centimeter (table 2). It is, of course, impossible to state whether this reduction in the number of viable organisms is altogether the result of the bactericidal action of the bacteriolysant or whether inhibition does not also play a part. Several cultures which

had been attacked in varying degrees by filtrates were centrifuged and the sediment stained. The amount of cellular debris was variable but the number of morphologically typical dysentery

TABLE 2

*Comparison of bacteriolytic activity of bacteriolysants with the bacterial counts of the culture before and after the action of bacteriolysants*

| 0.5 cc. of each of the following bacteriolysants were added to 2 cc. of an eight hour peptone water culture of Flexner Y, and then incubated together at 37°C. for twenty-four hours | Number of organisms per cubic centimeter in the eight hour peptone water culture of Flexner Y, before bacteriolysant was added | Degree of lysis after culture and bacteriolysant were incubated together at 37°C. for twenty-four hours | Number of viable organisms per cubic centimeter after culture and bacteriolysant were incubated together at 37°C. for twenty-four hours |
|---|---|---|---|
| F 24(3)A | 100,000,000 + | + | 264 |
| F 49(2) | 100,000,000 + | + | 44 |
| F 43(1) | 100,000,000 + | ++ | 2 |
| F 44 | 100,000,000 + | + | 130 |
| (Control) 1 per cent sterile peptone water | 100,000,000 + | 0 | 100,000,000 + |

TABLE 3

*Comparison of the degree of bacteriolysis with appearance of the stained sediment*

| 0.5 cc. of each of the following bacteriolysants were added to 2 cc. of an eight hour peptone water culture of Flexner Y, and then incubated together at 37°C. for seventy-two hours | Degree of lysis after the culture and bacteriolysant were incubated together at 37°C. for seventy-two hours | Appearance of the stained sediment (Gram's stain) after the culture and bacteriolysant had been incubated together at 37°C. for seventy-two hours and then centrifuged | |
|---|---|---|---|
| | | Amorphorus cellular debris | Morphologically typical dysentery bacilli |
| F 24 (3) A | +++ | ± | ± |
| F 49 (2) | ++ | +++ | ± |
| F 34 (1) | ++ | ++ | ± |
| F 43 (1) | + | ± | ± |
| F 31 | + | ++ | ++ |
| F 45 | + | +++ | ++ |
| F 48 | + | +++ | ++ |
| F 44 | ± | + | +++ |
| (Control) 1 per cent sterile peptone water | 0 | 0 | +++ |

bacilli was inversely proportional to the amount of lysis that had occurred (table 3).

The bacteriolytic activity of these filtrates was also tested in several instances by dropping a small portion of the filtrate on the surface of an agar plate which had been heavily inoculated five to twenty-four hours previously with Flexner bacilli. These plates were than incubated for twenty-four hours and the presence or absence of macroscopic lysis in the area bathed by the filtrate was noted. The presence of "moth eaten" colonies in subcultures of these areas confirmed the occurrence of lysis.

### ORGANISMS ATTACKED BY STOOL BACTERIOLYSANTS

Five stock laboratory Flexner strains, twenty freshly isolated Flexner strains, one stock laboratory Shiga strain and one stock laboratory typhoid strain were all lysed by one or more of sixty-eight bacteriolysants (either original stool filtrates or their subsequent generations) obtained from eleven patients (table 1).

### VARIATIONS IN THE BACTERIOLYTIC ACTIVITY OF STOOL BACTERIOLYSANTS

All of the sixty-eight bacteriolysants, some of which were tested against as many as eleven among this total of twenty-seven strains were active against one or more strains. Some filtrates attacked several strains, others only one. Among the two hundred and twenty-five lysis tests performed with these sixty-eight filtrates and twenty-seven strains, twenty-five tests were negative.

Among fifty filtrates which were tested against two to eleven different strains, fifteen (or 30 per cent) had the same titre of lysis against all of the organisms against which they were tested, i.e., filtrate no. 31 (4) when diluted 1:21 lysed all seven of the strains of the Flexner bacillus against which it was tested. Thirty-five filtrates (or 70 per cent) produced lysis in different dilutions, i.e., filtrate no. 33 (1) lysed six strains at a dilution of 1:21 and one strain at 1:5. The bacteriolytic titre of eight filtrates was tested against four dysentery cultures obtained by fishing four separate colonies from the same plate of a stool culture. Six of these filtrates lysed all four cultures at a dilution of 1:21 while the seventh filtrate lysed one culture at 1:21, two cultures

at 1:9 and one at 1:5 and the eighth filtrate attacked two cultures at 1:21 and two at 1:5. It is thus obvious that cultures differ in "lysability" just as they vary in "agglutinability." One Flexner strain was tested with forty-eight different bacteriolysants, and was lysed by thirty-nine at a dilution of 1:21, by three at a dilution of 1:9, by five at a dilution in 1:5 and was not lysed at all by one.

The bacteriolytic power of subsequent generations of a stool filtrate, obtained by filtering a dysentery culture which had been lysed by being in contact for twenty-four hours or more with a stool filtrate, in some instances was the same, in others greater and in others less than that of the original filtrate. The type of dysentery bacillus to which the original filtrate was added in the preparation of these subsequent generations, apparently did not affect the titre of the succeeding generation of filtrate. Among four filtrates that were carried through three to six generations and were always tested against the same organism, two had the same titres of lysis after each transfer and two had different titres, i.e., after one "passage" lytic action might be increased and after another "passage" it might be decreased. Among three filtrates that were carried three to ten generations and were always tested against two or more organisms, the titre of lysis in each generation remained the same for one organism and was different (increased or decreased) for the others.

Twelve first to sixth generation filtrates, the originals of which were obtained from the stools of four dysentery patients, were tested against the dysentery bacillus isolated from those patients' stools as well as against other strains of Bact. dysenteriae. Four of these filtrates were more active against their own patient's organism than the other strains, four were less active and four were equally active.

The bacteriolytic power of filtrates that had become contaminated with stool or air organisms and were then refiltered, was sometimes the same, sometimes greater and sometimes less than that of the original filtrate. Among nine contaminanted filtrates that were refiltered three had increased lytic power, three had decreased activity and in three the activity was unchanged.

Among thirteen filtrates heated immediately after filtration to 60 to 67°C. for forty-five to sixty minutes, the titre of lysis was reduced in five, apparently increased in two and unchanged in six. The changes were however so slight as to be within the range of experimental error, so that it is probable that the bacteriolytic power of a filrate is unaffected by these temperatures.

The degree of lysis is also dependent upon the concentration of the bacteriolytic filtrate, lysis frequently occurring at a dilution of 1:5 and being absent at 1:9, or 1:21.

### LOSS OF BACTERIOLYTIC ACTIVITY WHEN A BACTERIOLYSANT IS SUCCESSIVELY SUBCULTURED IN STERILE PEPTONE WATER OR IN A CULTURE KILLED BY HEAT

A bacteriolysant was added to a flask of sterile peptone water incubated twenty-four hours at 37°C. and then filtered. A portion of this filtrate was then added to another flask of sterile peptone water, incubated twenty-four hours at 37°C. and then filtered. A portion of this filtrate was then added to another flask of sterile peptone water, incubated twenty-four hours and then filtered. This was repeated for six such "passages." The bacteriolytic activity which was marked in the original bacteriolysant became progressively weaker and disappeared after the fifth passage, i.e., when the original bacteriolysant was diluted 1:7776. Although undemonstrable in the fourth "passage" when the original bacteriolysant was diluted 1:1296, yet subcultures of the dysentery bacilli which had been incubated with this filtrate contained a few "sensitive" colonies so that a slight amount of lytic activity was probably present at that dilution (table 4).

The effect of filtration on the bacteriolytic activity of these preparations was also studied, i.e., after the bacteriolysant had been incubated with sterile peptone water, the bacteriolytic activity of a portion of it was tested. The remainder was then filtered and the bacteriolytic activity of this filtrate tested. The titre of lysis of the unfiltered and the filtered portions was the same.

TABLE 4

*Loss of bacteriolytic activity when bacteriolysant is successively "subcultured" in sterile peptone water*

| FILTRATE USED | BACTERIOLYTIC ACTIVITY OF FILTRATE AGAINST CULTURES OF NORMAL FLEXNER DYSENTERY BACILLI | PRESENCE OF "SENSITIVE" COLONIES IN AGAR SUBCULTURES OF DYSENTERY BACILLI AFTER THEY HAD BEEN INCUBATED WITH THE FILTRATE FOR TWENTY-FOUR HOURS AT 37°C. | DILUTION OF ORIGINAL BACTERIOLYSANT IN PEPTONE WATER FILTRATE |
|---|---|---|---|
| *Original bacteriolysant* | ++ | Not subcultured | |
| *First "passage" in sterile peptone water,* i.e., 20 cc. of original bacteriolysant added to 100 cc. sterile peptone water, incubated twenty-four hours at 37°C. and then filtered | ± | ++ | 1:6 |
| *Second "passage" in sterile peptone water,* i.e., 20 cc. of 1st "passage" filtrate added to 100 cc. sterile peptone water, incubated twenty-four hours at 37°C. and then filtered | Doubtful | + | 1:36 |
| *Third "passage" in sterile peptone water,* i.e., 20 cc. of second "passage" filtrate added to 100 cc. sterile peptone water, incubated twenty-four hours at 37°C. and then filtered | Very doubtful | + | 1:216 |
| *Fourth "passage" in sterile peptone water,* i.e., 20 cc. of third "passage" filtrate added to 100 cc. sterile peptone incubated twenty-four hours at 37°C. and then filtered | 0 | ± | 1:1296 |
| *Fifth "passage" in sterile peptone water,* i.e., 20 cc. of fourth "passage" filtrate added to 100 cc. sterile peptone water, incubated twenty-four hours at 37°C. and then filtered | 0 | 0 | 1:7776 |
| *Sixth "passage" in sterile peptone water,* i.e., 20 cc. of fifth "passage" filtrate added to 100 cc. sterile peptone water incubated twenty four hours at 37°C. and then filtered | 0 | 0 | 1:46,656 |

An eighteen-hour peptone water culture of normal Flexner bacilli was killed by being heated to 60 to 65°C. for one hour (table 5). A bacteriolysant was then added to this dead culture and the preparation incubated twenty-hour hours at 37°C. and then filtered. This filtrate would not produce macroscopic lysis, but subcultures of the dysentery bacilli to which the filtrate was added contained a few "sensitive" colonies so that a certain amount of lytic activity was probably present. However this amount was less than that contained in a bacteriolysant after "passage" through sterile peptone water and almost negligible when compared with the bacteriolytic activity of a bacteriolysant after "passage" through a live culture of Flexner bacilli (table 5). The slight amount of bacteriolytic activity that this filtrate contained is probably due to the fact that there was sufficient of the original bacteriolysant present to cause the production of "sensitive" colonies. The fact that "passage" through a dead culture reduced the bacteriolytic activity of the bacteriolysant more than "passage" through the same amount of sterile peptone water suggests that the dead bacteria may have adsorbed the lytic principle in much the same way that kaolin adsorbs enzymes.

### THE EFFECT OF THE REACTION OF THE MEDIA IN WHICH DYSENTERY BACILLI WERE GROWN, ON THE DEGREE OF LYSIS PRODUCED IN A CULTURE BY A BACTERIOLYSANT

Flasks of 1 per cent peptone water, to which phenol-sulphonephthalein was added, were adjusted to each of the following reactions: pH 6.0, 6.6, 7.1, 7.4, 7.7, 8.0 and 8.2. Two cubic centimeters of the media at each reaction were placed in several tubes and these were sterilized in the autoclave. These tubes were then inoculated with Flexner dysentery bacilli. After four to twenty-four hours' incubation, bacteriolysants were added and the degree of lysis produced was noted. As may be seen in table 6, cultures whose initial pH was 8.0 and 8.2 were lysed somewhat more completely than cultures whose reactions were from pH 6.0 to 7.7.

TABLE 5

*Comparison of effect on bacteriolytic activity of a bacteriolysant of "subculturing" it in a dead culture of normal Flexner bacilli, in a live culture of normal Flexner bacilli and in sterile peptone water*

| FILTRATE USED | BACTERIOLYTIC ACTIVITY OF FILTRATE AGAINST CULTURES OF NORMAL FLEXNER DYSENTERY BACILLI | PRESENCE OF "SENSITIVE" COLONIES IN AGAR SUBCULTURES OF DYSENTERY BACILLI AFTER THEY HAD BEEN INCUBATED WITH THE FILTRATE FOR TWENTY-FOUR HOURS AT 37°C. | DILUTION OF THE ORIGINAL BACTERIOLYSANT IN "PASSAGE" FILTRATE |
|---|---|---|---|
| Original bacteriolysant | ++ | Not subcultured | |
| *Filtrate after "passage" in dead culture of Flexner dysentery bacilli*, i.e., 20 cc. of original bacteriolysant added to 100 cc. of *dead* eighteen-hour peptone water culture of normal Flexner bacilli (*killed by being heated to 60 to 65°C. for one hour*), incubated twenty-four hours at 37°C. and then filtered | 0 | + | 1:6 |
| *Filtrate after "passage" in live culture of Flexner dysentery bacilli*, i.e., 20 cc. of original bacteriolysant added to 100 cc. of an eighteen-hour peptone water culture of normal Flexner bacilli, incubated twenty-four hours at 37°C. and then filtered | +++ | ++ | 1:6 |
| *Filtrate after "passage" in sterile peptone water*, i.e., 20 cc. of original bacteriolysant added to 100 cc. of sterile peptone water, incubated twenty-four hours at 37°C. and then filtered | ± | ++ | 1:6 |

## COMPARISON OF BACTERIOLYTIC ACTIVITY OF BACTERIOLYSANT AGAINST SALINE AND PEPTONE WATER SUSPENSIONS OF NORMAL DYSENTERY BACILLI

Bacteriolysants were equally active against normal dysentery bacilli suspended in 0.9 per cent saline and 1 per cent peptone water provided the reactions of the two suspensions were the same (table 7). It is possible that earlier reports (Davison, 1922) of the failure of bacteriophages to lyse saline suspensions of dysentery bacilli may be explained by the fact that the reaction of the saline used, was too far from the optimum.

TABLE 6

*The effect of the reaction of the media in which dysentery bacilli were grown, on the degree of lysis produced in a culture by a bacteriolysant*

| BACTERIOLYSANT USED | FLEXNER DYSENTERY CULTURE USED IN LYSIS TESTS | INITIAL pH OF THE DYSENTERY CULTURE USED IN LYSIS TESTS | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 6.0 | 6.6 | 7.1 | 7.4 | 7.7 | 8.0 | 8.2 |
| F 34(3)B ... | Four-hour peptone water culture of Flexner Y (Hiss and Russell) | ± | + | + | + | + | + | ++ |
| F 42(2)..... | Four hour peptone water culture of Flexner 120 (isolated from patient in H.L.H.) | ± | ± | ± | ± | ± | + | + |
| F 48(3)..... | Twenty-four hour peptone water culture of Flexner 90 (isolated from patient in H.L.H.) | + | + | + | + | + | + | ++ |

## "RESISTANCE" OF OLD PEPTONE WATER CULTURES TO BACTERIOLYSANTS

As shown in table 8, bacteriolysants had no bacteriolytic activity against a one hundred and thirty-day-old peptone water culture of normal Flexner Y bacilli. There are at least four explanations for this failure, i.e., (1) that the reaction of the culture was too far from the optimum, (2) that the culture contained so many organisms that the lytic principle was adsorbed by the bacterial bodies, (3) that the organisms were all "resistant" and (4) that the culture was dead. However, its reaction was pH 8.0 which is at or near the optimum. The concentra-

tion factor did not appear to be essential for when the culture was diluted with saline to the same opacity as a three hour culture it was not lysed. That the organisms were not all dead and "resistant" was demonstrated by the fact that subcultures of this old culture grew and also contained a few "irregular" colonies. The fact that the culture when diluted with 1 per cent peptone water was lysed by the bacteriolysant may be explained by the fact that the organisms grew in the peptone

TABLE 7

*Comparison of bacteriolytic activity of bacteriolysant against saline and peptone water suspensions of agar cultures of normal dysentery bacilli (Flexner Y, Hiss and Russell)*

| BACTERIOLYSANT USED | BACTERIAL SUSPENSION USED TO TEST BACTERIOLYTIC ACTIVITY | BACTERIOLYTIC ACTIVITY OF BACTERIOLYSANT AGAINST THE BACTERIAL SUSPENSION |
|---|---|---|
| F 31(4)..... | Growth from seven-hour agar culture of normal Flexner bacilli suspended in 0.9 per cent saline at pH 8.0 | + |
| F 34(2)B..... | Growth from seven-hour agar culture of normal Flexner bacilli suspended in 0.9 per cent saline at pH 8.0 | + |
| F 31(4)..... | Growth from seven-hour agar culture of normal Flexner bacilli suspended in 1 per cent peptone water at pH 8.0 | + |
| F 34(2)B..... | Growth from seven-hour agar culture of normal Flexner bacilli suspended in 1 per cent peptone water at pH 8.0 | + |

water which was added, and it was then a young culture that was being attacked. The most probable explanation for the greater bacteriolytic activity of bacteriolysants when tested against young cultures, is that many of the organisms in any culture older than twenty-four hours, are dead and act like kaolin in enzymatic phenomena and adsorb the bacteriolytic principle.

### EFFECT OF SODIUM HYDROXIDE UPON A BACTERIOLYSANT

One cubic centimeter of normal sodium hydroxide was added to 4 cc. of a bacteriolysant and after eighteen hours incubation

at 37°C. the reaction was adjusted to pH 8.0 with normal hydrochloric acid (0.82 cc.). The bacteriolysant after this treatment had no bacteriolytic power, i.e., sodium hydroxide in a

TABLE 8

*Bacteriolytic activity of bacteriolysants against old peptone water cultures of normal dysentery bacilli (Flexner Y, Hiss and Russell)*

| BACTERIOLYSANT USED | CULTURE USED TO TEST BACTERIOLYTIC ACTIVITY | BACTERIOLYTIC ACTIVITY OF BACTERIOLYSANT AGAINST THE CULTURE TESTED |
|---|---|---|
| F 43(5)..... | One hundred and thirty-day peptone water culture of Flexner Y (undiluted) | 0 |
| F 34(3)B..... | One hundred and thirty-day peptone water culture of Flexner Y (undiluted) | 0 |
| F 43(5)..... | One hundred and thirty-day peptone water culture of Flexner Y (diluted ten times with 0.9 per cent saline) (to same opacity as a three-hour culture) | 0 |
| F 34(3)B..... | One hundred and thirty-day peptone water culture of Flexner Y (diluted ten times with 0.9 per cent saline) (to same opacity as a three-hour culture) | 0 |
| F 43(5)..... | One hundred and thirty-day peptone water culture of Flexner Y (diluted ten times with 1 per cent peptone water) (to same opacity as a three-hour culture) | ± |
| F 34(3)B..... | One hundred and thirty-day peptone water culture of Flexner Y (diluted ten times with 1 per cent peptone water) (to same opacity as a three-hour culture) | ± |
| F 43(5)..... | Three-hour peptone water culture of Flexner Y | ++ |
| F 34(3)B..... | Three-hour peptone water culture of Flexner Y | ++ |

strength of N/5 destroyed the lytic principle, normal sodium hydroxide killed dysentery bacilli and also lysed the organisms. Twenty per cent hydrochloric acid killed the culture but did not lyse it (table 9). The organisms treated with hydrochloric acid did not stain well, however.

TABLE 9

*Effect of sodium hydroxide upon a bacteriolysant*

| BACTERIOLYSANT OR CHEMICAL USED | BACTERIOLYTIC ACTIVITY OF BACTERIOLYSANT OR CHEMICAL AGAINST CULTURES OF NORMAL FLEXNER BACILLI | PRESENCE OF "SENSITIVE" COLONIES IN AGAR SUBCULTURES OF DYSENTERY BACILLI AFTER THEY HAD BEEN INCUBATED WITH THE BACTERIOLYSANT OR CHEMICAL FOR TWENTY-FOUR HOURS AT 37°C. |
|---|---|---|
| F 34(3)B, after incubation for eighteen hours with N/5 NaoH and then adjusted to pH 8.0 with N/1 HCl | 0 | Not subcultured |
| F 34(3)B (without NaOH or HCl) | + | + |
| N/1 NaOH | + | No growth on subculture, i.e., culture killed |
| 20 per cent HCl | 0 | No growth on subculture, i.e., culture killed |

## RESULTS OF INOCULATIONS OF BACTERIOLYTIC FILTRATES INTO ANIMALS

Four doses of 5 to 15 cc. of a bacteriolytic filtrate at intervals of six to seventy-five days were injected intravenously and subcutaneously into a rabbit. The animal showed no ill effects. Its serum after the fourth injection agglutinated three strains of Flexner bacilli in a dilution of 1:250 and would not agglutinate Bact. coli at that dilution. Precipitin tests with this rabbit's serum and a bacteriolytic filtrate were positive in a dilution of 1:2, and negative at 1:20.

## SUMMARY

The filtrates of the stools of infants suffering from bacillary dysentery (Flexner), acute intestinal indigestion, otitis media and the need for regulation of feeding were bacteriolytic for one or more of twenty-seven strains of Flexner Shiga and typhoid bacilli. The technique by which these filtrates were obtained and tested, is described. D'Herelle's phenomenon is apparently non-specific and a bacteriolysant from a patient's stool may not be as active against the organism causing that patient's disease as against other strains. It does not necessarily play a part in the immunity or defense-mechanism of the body for

an active bacteriolysant was obtained from the stool of a patient the day before his death. One cannot at present predict which organisms will be attacked by any given filtrate, nor explain why certain cultures are readily lysed while others are unaffected. The bacteriolytic activity of a bacteriolysant may or may not be increased by passage through live dysentery cultures but it is decreased by passage through sterile peptone water and dead dysentery bacilli. Contamination and refiltration may increase or decrease the potency of a filtrate. Heating to 60 to 67°C. for forty-five to sixty minutes has little or no effect upon the activity of a filtrate. Bacteriolysants produce lysis more completely in low dilutions and at a pH of 8.0 and 8.2. Saline and peptone water suspensions of Flexner bacilli at pH 8 were equally lysable. Young cultures are lysed more readily than older ones. The addition of 1 cc. of N sodium hydroxide to 4 cc. of a bacteriolysant destroyed its bacteriolytic activity. Bacteriolysants were non-pathogenic for a rabbit and as I have previously reported (Davison, 1922b) had no therapeutic effect when administered to twelve young children suffering from bacillary dysentery.

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# OBSERVATIONS ON THE NATURE OF BACTERIOLYSANTS (D'HERELLE'S PHENOMENON, BACTERIOPHAGE, BACTERIOLYTIC AGENT, ETC.)

## PART II[1]

WILBURT C. DAVISON
*Baltimore, Maryland*

From a consideration of the properties of bacteriolytic filtrates of stool cultures, which I have discussed in part I as well as in a review of the whole subject (Davison, 1922) it would seem possible that the bacteriolytic principle might be an enzyme, although as d'Herelle (Davison, 1922) has pointed out, none of the evidence absolutely disproves his contention that a bacteriophage is a living ultra-microscopic filter-passing organism. If then, one assumes that the substance is enzymatic, and that it can only be propagated from generation to generation by passage through living cultures one must conclude that it is contained in the bacterial cells or is a product of their metabolism. I therefore studied the properties of the colonies of "sensitive" and "normal" strains of Bact. dysenteriae (Flexner).

### STUDY OF THE COLONIES OF A "SENSITIVE" STRAIN OF BACT. DYSENTERIAE (FLEXNER)

One-tenth cubic centimeter of a bacteriolysant was dropped on the surface of a five-hour agar culture of normal Flexner Y bacilli (Hiss and Russell) and the plate was replaced in the incubator at 37°C., for twenty-four hours. At the end of this time, the area bathed by the bacteriolysant was apparently devoid of growth or else covered with a fine film, while the remainder of the plate

[1] Presented at the twenty-third annual meeting, Society of American Bacteriologists, December 29, 1921. From the Department of Pediatrics of the Johns Hopkins University and the Harriet Lane Home of the Johns Hopkins Hospital, Baltimore, Maryland.

was covered with typical round dysentery colonies. This "bare" area was subcultured on another agar plate. After twenty-four hours at 37°C. this plate contained in equal proportions, small, regular, round, typical dysentery colonies and large mucoid colonies with very irregular outlines and a "moth eaten" appearance. Some were crescentic in shape, others triangular, and still others somewhat round with deep indentations in the edges. The edges of many of these colonies were more dense and refractile than their centers and appeared white and opaque in contrast to the gray translucency of the center. They resembled in every way, the photographs of "sensitive" colonies that Wollstein (Davison, 1922) has published. Forty-two successive subcultures of these "moth eaten" colonies, each gave rise to 10 to 100 per cent of the "moth eaten" colonies and 0 to 90 per cent of the small regular round colonies. Press of work prevented carrying these subcultures to more than forty-two generations.

Occasionally subcultures of the "moth eaten" colonies failed to grow in peptone water or on agar and reinoculation with several "moth eaten" colonies was necessary. When a "moth eaten" colony was subcultured in peptone water for one or two transfers, and then plated on agar, the plates frequently remained sterile or else contained nothing but regular colonies indicating the influence that a change in media might play in the multiplication of one or the other of these types. Gram-stained smears of these "moth eaten" colonies were largely composed of short fat Gram-negative bacilli with very few long forms. They were decolorized with more difficulty than smears of normal colonies. The "moth eaten" colonies represent the "sensitive" or "lysogenic" strain and the small, regular, round colonies the "resistant" strain described by others (Davison, 1922).

### FILTRATES OF PEPTONE WATER CULTURES OF A "SENSITIVE" STRAIN OF BACT. DYSENTERIAE (FLEXNER)

The growth from twelve "moth eaten" colonies of the eighth generation of the "sensitive" strain of Flexner bacilli described above was inoculated into 500 cc. of peptone water (table 10).

This culture was then incubated at 37°C. for eighteen hours and 150 cc. of it was filtered. This filtrate was strongly bacteriolytic for normal Flexner bacilli. The remaining 350 cc. was then centrifuged at high speed and the supernatant fluid filtered. This filtrate was also strongly bacteriolytic. The sediment of

TABLE 10

*Bacteriolytic activity of filtrates of peptone water cultures of a "sensitive" strain of Bact. dysenteriae (Flexner Y, Hiss and Russell)*

| FILTRATE OR SUSPENSION USED | BACTERIOLYTIC ACTIVITY OF FILTRATE AGAINST CULTURES OF NORMAL FLEXNER BACILLI | PRESENCE OF "SENSITIVE" COLONIES IN AGAR SUBCULTURES OF DYSENTERY BACILLI AFTER THEY HAD BEEN INCUBATED WITH THE FILTRATE FOR TWENTY-FOUR HOURS AT 37°C. |
|---|---|---|
| Filtrate of eighteen-hour peptone water culture of the eighth generation of a "sensitive" strain of Flexner bacilli | + | ++ |
| Filtrate of supernatant fluid of an eighteen-hour peptone water culture of the eighth generation of a "sensitive" strain of Flexner bacilli after the organisms had been centrifuged out | + | ++ |
| Saline suspension of the organisms of an eighteen-hour culture of the eighth generation of a "sensitive" strain of Flexner bacilli, after they had been centrifuged out and washed twice with saline. This suspension had stood at room temperature for 35 days to allow the organisms to disintegrate | ± | ++ |
| Filtrate of ninety-six-hour peptone water culture of the thirty-fourth generation of a "sensitive" strain of Flexner bacilli | + | ++ |
| Sterile 1 per cent peptone water (control) | 0 | 0 |

organisms was washed twice with 15 cc. of 0.9 per cent saline and then suspended in 5 cc. of saline. This suspension was left at room temperature for thirty-five days. Subcultures were then sterile. This suspension was also bacteriolytic. In other words, the lytic principle is both extracellular and intracellular for the fluid medium in which a "sensitive" strain was grown and also

the saline in which the washed organisms had been suspended and then allowed to disintegrate, were both bacteriolytic.

The growth from several "moth eaten" colonies of the thirty-fourth generation of the "sensitive" strain described above was inoculated into 100 cc. of peptone water. This culture was incubated ninety-six hours at 37°C. and then filtered. This filtrate was as strongly bacteriolytic as that of the eighth generation. This bears out Bordet's and Ciuca's (Davison, 1922) statement that "sensitive" strains are lysogenic for numerous generations.

FILTRATES OF AGAR CULTURES OF A "SENSITIVE" AND A NORMAL STRAIN OF BACT. DYSENTERIAE (FLEXNER)

The twenty-four hours' growth of "moth eaten" colonies on five agar plates of the fifth generation of the "sensitive" strain of Flexner bacilli described above was suspended in 20 cc. of N/10 phosphate solution at pH 8[2] and centrifuged. The supernatant phosphate solution was then filtered. This filtrate was strongly bacteriolytic (table 11). The sediment of organisms, which was a mass of mucoid, stringy material, was resuspended in 20 cc. of N/10 phosphate solution and ground up in a rotary agate mortar.[3] This suspension was then filtered. This filtrate was also strongly bacteriolytic (table 11). In other words the phosphate solution in which the sensitive organisms were washed and also the solution which contained the products of the ground up organisms were both bacteriolytic, suggesting that the lytic principle was extracellular as well as intracellular.

The twenty-four hours' growth of regular colonies on nine agar plates of a normal strain of Flexner bacilli was suspended in 20 cc. of N/10 phosphate solution at pH 8 and ground up in a rotary agate mortar. This suspension was then filtered. This filtrate had no bacteriolytic activity (table 11).

[2] N/10 $Na_2HPO_4$, 1.95 parts, and N/10 $KH_2PO_4$, 0.05 part. This solution itself was not bacteriolytic.

[3] The grinding was done by Dr. L. B. Lange of the Johns Hopkins School of Hygiene, to whom my thanks are due.

An attempt was made to break up saline suspensions of the twenty-four hours' growth on agar of Flexner bacilli and also of B. subtilis by alternate freezing and thawing but though the suspensions were frozen and thawed once or twice a day for thirty-five days, smears and cultures indicated that no damage

TABLE 11

*Comparison of the bacteriolytic activity of filtrates of agar cultures of a "sensitive" and a normal strain of Bact. dysenteriae (Flexner Y, Hiss and Russell) suspended in N/10 phosphate solution of pH 8 and ground in a rotary agate mortar*

| FILTRATE USED | BACTERIOLYTIC ACTIVITY OF FILTRATE AGAINST CULTURES OF NORMAL FLEXNER BACILLI | PRESENCE OF "SENSITIVE" COLONIES IN AGAR SUBCULTURES OF DYSENTERY BACILLI AFTER THEY HAD BEEN INCUBATED WITH THE FILTRATE FOR SEVENTY-TWO HOURS AT 37°C. |
|---|---|---|
| Filtrate of the N/10 phosphate solution in which the growth from a twenty-four-hour agar culture of the fifth generation of a "sensitive" strain had been washed, i.e., in which the organisms had been suspended and then centrifuged out | + | ++ |
| Filtrate of the N/10 phosphate solution suspension of the washed organisms of a twenty-four hour agar culture of the fifth generation of a "sensitive" strain after they had been ground up in a rotary agate mortar | + | ++ |
| Filtrate of the N/10 phosphate solution suspension of the unwashed organisms of a twenty-four-hour agar culture of a *normal* strain of Flexner bacilli after they had been ground up in a rotary agate mortar | 0 | 0 |
| N/10 phosphate solution of pH 8 (control) | 0 | 0 |

had been done to the organsism. This is in marked contrast to the death of "sensitive" bacilli suspended in saline, at room temperature for thirty-four days (vide supra). Attempts were also made to dissolve dysentery bacilli by the addition of sodium hydroxide and of trypsin but the amount of the former required was sufficient to destroy any lytic activity that might have been present (table 9 part I,) and trypsin would not dissolve the organisms (table 15).

TABLE 12

*The occurrence of "irregular" colonies in platings of normal strains of Bact. dysenteriae (Flexner)*

| STRAIN OF BACT. DYSENTERIAE (FLEXNER) SUBCULTURED | AGE AND MEDIA OF CULTURE WHICH WAS SUBCULTURED | NUMBER OF "IRREGULAR" COLONIES IN SUBCULTURE PLATE AFTER TWENTY-FOUR HOURS AT 37°C. | NUMBER OF GENERATIONS TO WHICH "IRREGULAR" COLONY WAS CARRIED |
|---|---|---|---|
| Flexner Y (Hiss and Russell) (obtained from the Lister Institute, London | 3-hour peptone water culture | Several | Subculture contained 1 "irregular" colony; not carried farther |
| Flexner Y (Hiss and Russell) (obtained from the Lister Institute, London | 4-day peptone water culture | 0 | |
| Flexner Y (Hiss and Russell) (obtained from the Lister Institute, London) | 136-hour peptone water culture | 1 | Subculture contained no "irregular" colonies |
| Flexner Y (Hiss and Russell) (obtained from the Lister Institute, London) | 145-hour peptone water culture | 0 | |
| Flexner Y (Hiss and Russell) (obtained from the Lister Institute, | 153-hour peptone water culture | 1 | Subculture contained 2 "irregular" colony; not carried farther |
| Flexner Y (Hiss and Russell) (obtained from the Lister Institute, London) | 6-day peptone water culture (containing 1 per cent lactose) | Several | Subculture contained no "irregular" colonies |
| Flexner Y (Hiss and Russell) (obtained from the Lister Institute, London) | 43-day peptone water culture (containing 1 per cent lactose) | Several | Subculture contained several "irregular" colonies; not carried farther |
| Flexner Y (Hiss and Russell) (obtained from the Lister Institute, London) | 27-day agar culture | Several | Two successive subcultures each contained 2 "irregular" colonies; not carried further |

| | | | |
|---|---|---|---|
| Flexner Y (Ledingham) (obtained from Dr. E. G. D. Murray, London) | 55-day peptone water culture | 2 | Subculture contained no "irregular" colonies |
| Flexner Y (Ledingham) (obtained from Dr. E. G. D. Murray, London) | 58-day peptone water culture | 0 | |
| Flexner Y (Ledingham) (obtained from Dr. E. G. D. Murray, London) | 6-day peptone water culture (containing 1 per cent lactose) | Several | Nine successive subcultures each contained several "irregular.' colonies |
| Flexner Y (Ledingham) (obtained from Dr. E. G. D. Murray, London) | 42-day peptone water culture (containing 1 per cent lactose) | Several | Two successive subcultures each contained several "irregular" colonies; not carried farther |
| Flexner Y (Ledingham) (obtained from Dr. E. G. D. Murray, London) | 26-day agar culture | 1 | Two successive subcultures each contained several "irregular" colonies; not carried farther |
| Flexner Y (Ledingham) (obtained from Dr. E. G. D. Murray, London) | 20 (?)-day agar culture | Several | Not subcultured |
| Flexner Y (Sada Gopall) (obtained from Dr. E. G. D. Murray, London) | 55-day peptone water culture | Several | Two successive subcultures each contained several "irregular" colonies |
| Flexner Y (Sada Gopall) (obtained from Dr. E. G. D. Murray, London | 62-day peptone water culture | Several | Three successive subcultures each contained several "irregular" colonies |
| Flexner Y (Sada Gopall) (obtained from Dr. E. G. D. Murray, London) | 6-day peptone water culture (containing 1 per cent lactose) | Several | Ten successive subcultures each contained several "irregular" colonies; not carried farther |
| Flexner Y (Sada Gopall) (obtained from Dr. E. G. D. Murray, London) | 26-day agar culture | Several | Three successive subcultures each contained several "irregular" colonies; not carried farther |
| Flexner Y (Sada Gopall) (obtained from Dr. E. G. D. Murray, London) | 20 (?)-day agar culture | Several | Not subcultured |

TABLE 12—*Continued*

| STRAIN OF BACT. DYSENTERIAE (FLEXNER) SUBCULTURED | AGE AND MEDIA OF CULTURE WHICH WAS SUBCULTURED | NUMBER OF "IRREGULAR" COLONIES IN SUBCULTURE PLATE AFTER TWENTY-FOUR HOURS AT 37°C. | NUMBER OF GENERATIONS TO WHICH "IRREGULAR" COLONY WAS CARRIED |
|---|---|---|---|
| Flexner 115. (isolated frompatient in H. L. H.) | 20 (?)-day agar culture | Several | Not subcultured |
| Flexner 119. (isolated from patient in H. L. H.) | 20 (?)-day agar culture | Several | Not subcultured |
| Flexner 120. (isolated from patient in H. L. H.) | 20 (?)-day agar culture | Several | Subculture contained several "irregular" colonies |
| Flexner 90. (isolated from patient in H. L. H.) | 1-day peptone water culture | 0 | |
| Flexner 90. 123, 137, 139, 140 and 141. (Isolated from patients in H.L.H.) | 3-day peptone water culture | 0 | |
| Flexner W (stock culture); Flexner 120, and 131. (isolated from patients in H. L. H.) | 4-day peptone water culture | 0 | |
| Flexner 90, 107, 123, 129, 131 and 141. (isolated from patients in H.L.H.) | 20 (?)-day agar culture | 0 | |

STUDY OF THE COLONIES OF NORMAL STRAINS OF BACT. DYSENTERIAE (FLEXNER)

In sixteen out of twenty agar subcultures of four old laboratory strains of normal Flexner bacilli as well as in three out of eighteen agar subcultures of twelve freshly isolated strains of normal Flexner bacilli, I have noted one or more colonies with irregular

TABLE 13

*Bacteriolytic activity of filtrates of peptone water, cultures of normal dysentery bacilli (Flexner)*

| STRAIN OF FLEXNER DYSENTERY BACILLUS | TYPE OF COLONY | NUMBER OF CULTURES FILTERED | LENGTH OF INCUBATION AT 37°C. BEFORE FILTRATION | NUMBER OF ACTIVE FILTRATES | PERCENTAGE OF ACTIVE FILTRATES |
|---|---|---|---|---|---|
| | | | | | *per cent* |
| Flexner Y (Hiss and Russell) (obtained from the Lister Institute, London) | Regular | 8 | 5 hours to 152 days | 4 | 50 |
| Flexner Y (Ledingham) (obtained from Dr. E. G. D. Murray, London) | Regular | 5 | 1 to 91 days | 2 | 40 |
| | Irregular* | 2 | 1 day | 2 | 100 |
| Flexner Y (Sada Gopall) (obtained from Dr. E. G. D. Murray, London) | Regular | 5 | 1 to 91 days | 4 | 80 |
| | Irregular | 3 | 1 to 15 days | 3 | 100 |
| Flexner 106 (isolated from patient in H.L.H.) | Regular | 1 | 5 days | 1 | 100 |
| Flexner 120 (isolated from patient in H. L. H.) | Regular | 1 | 1 day | 0 | 0 |
| | Irregular | 1 | 1 day | 1 | 100 |
| Total | Regular | 20 | 5 hours to 152 days | 11 | 55 |
| | Irregular | 6 | 1 to 15 days | 6 | 100 |

* Colonies with irregular edges that are sometimes present in subcultures of normal strains.

edges among the mass of regular, round, typical, dysentery colonies (table 12). These colonies were not as "irregular" and "moth eaten" as those which were noted in subcultures of "sensitive" strains but nevertheless resembled them. Successive

subcultures of some of these spontaneously "irregular" colonies contained "irregular" colonies for as long as ten generations. Subcultures of others contained nothing but regular colonies. The "irregular" colonies did not appear to be as viable as normal colonies and subcultures occasionally failed to grow. Inasmuch as these "irregular" colonies occurred more frequently in subcultures of old laboratory strains than of freshly isolated strains, it is possible that aging may influence their development. Growth in media containing lactose also appeared to favor their development. Filtrates of cultures of these "irregular" colonies were somewhat more lytic than filtrates of normal cultures (table 13). It is possible that these "irregular" colonies of normal strains and the "moth eaten" colonies of "sensitive" strains are related or even perhaps identical.

The different degrees of lysis produced in different cultures by the same filtrate (part I) apparently did not depend altogether on the presence or absence of "irregular" colonies in these cultures as only in six strains were "irregular" colonies noted.

### FILTRATES OF PEPTONE WATER CULTURES OF NORMAL STRAINS OF BACT. DYSENTERIAE (FLEXNER)

Inasmuch as the bacteriolytic activity of stool filtrates, which had become contaminated with stool or air organisms and had then been refiltered, was sometimes increased, it seemed possible that filtrates of normal cultures themselves might be bacteriolytic. I therefore inoculated regular and "irregular" colonies of several strains of normal Flexner bacilli into peptone water and filtered these cultures after various periods of incubation at 37°C. (table 13). Eleven of the twenty filtrates (55 per cent) of peptone water cultures of regular colonies of five normal strains of Flexner bacilli and all of the six filtrates (100 per cent) of "irregular" colonies were slightly bacteriolytic. The degree of bacteriolysis was much less than that of stool filtrates and of filtrates of cultures of "sensitive" strains. In none of the instances in which subcultures were made of dysentery bacilli which had been incubated with these filtrates of normal cultures were "sensitive" colonies found. It is therefore probable that although filtrates

of normal strains of dysentery bacilli were bacteriolytic their activity was extremely weak.

### BACTERIOLYTIC ACTIVITY OF FILTRATES OF A PEPTONE WATER CULTURE OF BACT. SUBTILIS

Nicolle (Davison, 1922) reported that cultures of B. subtilis and their filtrates, were bacteriolytic for several types of organisms. As he had not tested the bacteriolytic activity of B. subtilis against Flexner bacilli, I did so. As shown in table 14, the filtrate of a culture of B. subtilis (obtained from a contaminated plate) was not bacteriolytic for Flexner bacilli but the

TABLE 14

*Bacteriolytic activity of filtrates of peptone water cultures of B. subtilis*

| FILTRATE OR SUSPENSION USED | BACTERIOLYTIC ACTIVITY OF FILTRATE OR SUSPENSION AGAINST NORMAL FLEXNER BACILLI |
|---|---|
| Filtrate of four-day peptone water culture of B. subtilis | 0 |
| Filtrate of peptone water culture of normal dysentery bacilli which had been incubated forty-eight hours with the filtrate of B. subtilis culture (above) | ± |
| Filtrate of eighteen-day peptone water culture or normal dysentery bacilli which had been intentionally contaminated with B. subtilis | ± |

filtrate of a normal dysentery culture to which the filtrate of a B. subtilis culture had been added, or which had been intentionally contaminated with B. subtilis, was slightly bacteriolytic. However this bacteriolytic activity was no more marked than that of filtrates of normal dysentery bacilli to which nothing had been added (table 13) so it is improbable that B. subtilis or its filtrates can lyse Flexner bacilli.

### ABSENCE OF BACTERIOLYTIC ACTIVITY IN TRYPSIN

As the optimum reaction for bacteriolysis apparently coincides with that of the enzyme, trypsin, I tested the bacteriolytic activity of solutions of trypsin and also compared the action on gelatin of trypsin, bacteriolysants and "sensitive" and normal strains of Flexner bacilli. 1 per cent and 5 per cent solutions of

trypsin (Fairchild and Foster) were made in normal saline and 20 per cent alcohol. These were proven sterile by culture. As

TABLE 15

*Absence of bacteriolytic activity in trypsin*

| SOLUTION OR FILTRATE USED | BACTERIOLYTIC ACTIVITY OF SOLUTION OR FILTRATE AGAINST CULTURES OF NORMAL DYSENTERY BACILLI | PRESENCE OF "SENSITIVE" COLONIES IN AGAR SUBCULTURES OF DYSENTERY BACILLI AFTER THEY HAD BEEN INCUBATED WITH SOLUTION OR FILTRATE FOR TWENTY-FOUR HOURS AT 37°C. |
|---|---|---|
| 1 per cent alcoholic solution of trypsin | 0 | 0 |
| 1 per cent saline solution of trypsin | 0 | 0 |
| 5 per cent alcoholic solution of trypsin | 0 | 0 |
| 5 per cent saline solution of trypsin | 0 | 0 |
| F 104 = 5 cc. 5 per cent alcoholic solution of Trypsin added to 100 cc. of an eighteen-hour peptone water culture of Flexner Y bacilli; incubated eighteen hours and then filtered | 0 | 0 |
| F 103 = 2 cc. 5 per cent saline solution of Trypsin, added to 100 cc. of an eighteen-hour peptone water culture of Flexner Y bacilli; incubated eighteen hours and then filtered | 0 | 0 |

TABLE 16

*Absence of gelatin liquefying enzyme in bacteriolysants and cultures of "sensitive" and "normal" strains of Bact. dysenteriae (Flexner Y) (Hiss and Russell)*

| BACTERIOLYSANT, CULTURE OR SOLUTION USED | LIQUIFICATION OF GELATIN AT ROOM TEMPERATURE |
|---|---|
| F 100 (actively bacteriolytic filtrate) | 0 (34 days) |
| "Moth eaten" colonies of "sensitive" strain of Flexner Y bacilli | 0 (48 days) |
| Regular colonies of normal strain of Flexner Y bacilli | 0 (48 days) |
| 5 per cent alcoholic solution of trypsin | + (24 hours) |
| 5 per cent saline solution of trypsin | + (24 hours) |

shown in table 15, neither these solutions, nor filtrates of dysentery cultures which had been incubated with these solutions were bacteriolytic. That the trypsin used was active and could liquefy gelatin is shown in table 16.

### ABSENCE OF GELATIN LIQUEFYING ENZYME IN BACTERIOLYSANTS AND CULTURES OF "SENSITIVE" AND "NORMAL" STRAINS OF BACT. DYSENTERIAE (FLEXNER Y, HISS AND RUSSELL)

As shown in table 16, gelatin was not liquefied by a bacteriolysant nor by cultures or "moth eaten" colonies of a "sensitive" strain of Flexner bacilli nor by regular colonies of a normal strain.

### SUMMARY

"Moth eaten" or "sensitive" colonies were obtained in subcultures of dysentery bacilli which had been attacked by a bacteriolysant. They were subcultured for forty-two successive generations. The media in which "sensitive" strains of Flexner bacilli were grown, the solutions in which they were washed, and the suspensions of disintegrated or ground up "sensitive" bacilli, were all strongly bacteriolytic. In agar subcultures of old laboratory and freshly isolated normal strains of Flexner bacilli "irregular" colonies were occasionally found, which resembled the "moth eaten" colonies of "sensitive" strains. Filtrates of peptone water cultures of normal strains and expecially of their "irregular" colonies, were slightly bacteriolytic. B. subtilis was not bacteriolytic for Flexner bacilli. Trypsin was not bacteriolytic and bacteriolysants and "sensitive" and "normal" strains did not liquefy gelatin.

### CONCLUSION

It would seem possible that the lytic principle in d'Herelle's phenomenon is an enzyme. This enzyme is not trypsin. A small amount of the lytic principle is contained in, or produced by, normal strains of Flexner bacilli which have been out of contact with the human body for many years. It is possible that the amount of the bacteriolytic enzyme produced by a culture can be increased by aging, by growth in special media or by contact with external influences such as intestinal secretions, tissue extracts, leucocytes, etc. The action of these external influences is probably to favor the develoment of lysogenic organisms at the

expense of the non-lysogenic. This enzyme not only dissolves organisms but also favors the multiplication of bacteria which produce this enzyme. In this way the bacteriolytic principle is carried from generation to generation. It is highly improbable that this phenomenon represents a defense mechanism on the part of an animal against bacterial invasion.

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# CLOSTRIDIUM PUTRIFICUM (B. PUTRIFICUS BIENSTOCK), A DISTINCT SPECIES

GEORGE F. REDDISH AND LEO F. RETTGER

*From the Sheffield Laboratory of Bacteriology, Yale University*

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There has been much confusion regarding the identity of the organism which was discovered by Bienstock (1884) and which he named *Bacillus putrificus*. According to his own admission, Bienstock for a while worked with impure cultures of this anaerobe. However, he described its morphology and putrefactive properties, in his first publication (1884), and at a later date published a more complete description (1899).

Bienstock described his *B. putrificus* as a long, slender rod with a terminal spore. His photographs show both round and more or less oval spores. Some investigators have interpreted his phrase "baguette de tambour" and the word "Trommelschlägenform" as meaning literally *drumstick* and implying an oval form of the spore. This interpretation does not appear to the writers to be correct. In its sporulating stage *Clostridium tetani* is described quite generally in the literature as a drumstick form, and this term is generally applied to organisms possessing a round, terminal spore. Tissier and Martelly (1902) liken the spore of *C. putrificum* to that of *C. tetani*. Rettger (1906) also points out a strong resemblance, and further draws attention to the ease with which *C. putrificum* may, by morphology alone, be mistaken for the tetanus bacillus. However, certain writers have in recent years referred to *C. putrificum* as producing a terminal oval spore. Even so late a work as that of the British Medical Research Committee (1917) has included this organism among the putrefactive anaerobes which develop such spores. Some American writers have fallen into the same error. Sturges and Rettger (1919), on the other hand, state emphatically as the result of a continued study of several pure

strains which had been isolated by recently devised methods, that the spores of this organism are round.

The writers have employed the strains isolated by Sturges, in their morphological, biochemical and cultural studies of *C. putrificum*, and have corroborated the earlier observations of Rettger and those of Sturges and Rettger, particularly the points regarding morphology. In all of the stock strains ex-

FIG. 1. SHOWING RODS, CHAINS AND CHARACTERISTIC DRUM STICKS OF *C. putrificum*. INCUBATED 3 WEEKS AT 37°C. IN EGG-MEAT MEDIUM. FUCHSIN STAIN. × 1000

amined (four) the microscopic appearances of the organism were the same. The bacilli were long and slender, frequently slightly curved, and in the sporulated condition possessed a terminal, round spore which in the fully developed drumsticks appeared very large as compared with the thickness of the rods. The rods were as a rule longer and much more slender than those of *C. tetani* grown on the same medium (fig. 1). Colonies also are characteristic (fig. 2).

There has been considerable discussion as to whether *C. putrificum* attacks carbohydrates. Bienstock did not deal with this phase in his earlier work. Tissier and Martelly (1902) showed that it does not act upon glucose, or at most only slightly. In a later publication (1906) Bienstock confirms the observations of Tissier and Martelly, and of Rodella (1905), and holds that *C. putrificum* does not attack any of the carbohydrates, and that the minute quantities of acid formed in sugar media arise from the decomposition of protein. This organism occupies a unique position, therefore, among the anaerobes, in that it attacks proteins but not carbohydrates appreciably if at all. Bienstock's

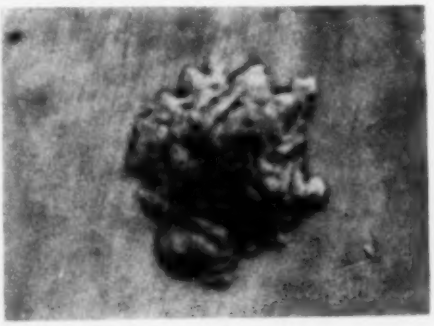

FIG. 2. COLONY ON 1.6 PER CENT GLUCOSE AGAR. INCUBATED 10 DAYS AT 37°C.

"*B. paraputrificus*," on the other hand, did attack carbohydrates with acid and gas formation.

In the recent reports of the British Medical Research Committee, *C. putrificum* is described as being saccharolytic to the extent of acting upon glucose, maltose, lactose, sucrose and starch. They even go further (1919) and consider it to be, not an entity or distinct species, but a mixture perhaps of *C. sporogenes* and *C. tertium* or of *C. sporogenes* and *C. cochlearius*.

In our own study of this organism its unique position appears to be so clearly defined and its characters are found to be so outstanding that we see no reason for mistaking it for any other anaerobe or mixture of anaerobes. Because of the fact that

the above mentioned work of the British Medical Research Committee has exerted and is exerting a commanding influence on present-day attempts at re-classification of the anaerobes, particularly those which were found in war wounds, we feel prompted to defend *C. putrificum* (*B. putrificus*) as a distinct species and to attempt to place it in its proper classification group.

*C. putrificum* bears a more or less close resemblance to *C. tetani*, and *C. tetanoides* morphologically, but differs markedly

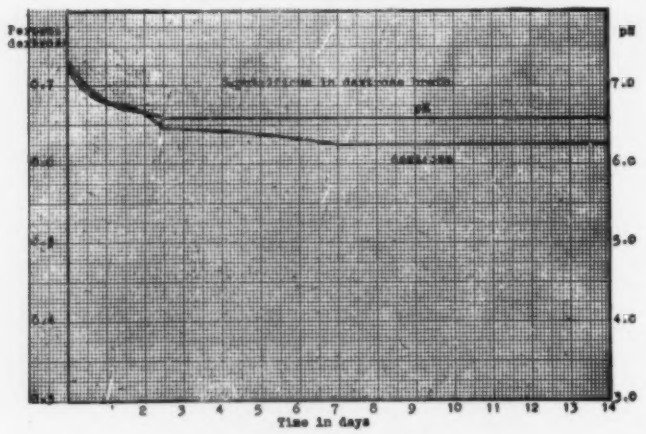


CHART 1. PLOTTINGS OF pH AND GLUCOSE FIGURES FOR *C. putrificum* IN GLUCOSE BROTH

from them in certain important respects. Pure strains of *C. tetani* and *C. tetanoides* are non-putrefactive, and are unable to digest meat or egg-meat medium even during months of incubation. *C. tetani* is pathogenic, whereas the other two organisms are entirely void of this property. Furthermore, *C. putrificum* does not attack carbohydrates or at the most exerts but a feeble action. No gas was produced from any of the 25 carbohydrates, alcohols and glucosides employed by us, and only slight amounts of acid from three or four, one of these being glucose. Quantitative estimations of the amounts of glucose consumed were made, and the results expressed in plotted curves (chart 1).

This organism constitutes a unique division, therefore, among the known anaerobes, and differs from all others in that it is powerfully putrefactive, but non-saccharolytic, or at the most but feebly saccharolytic.

While *C. putrificum* is an active proteolytic and putrefactive organism, it differs from *C. sporogenes* in that in pure culture in meat or egg-meat medium it develops slowly and produces little or no apparent change in less than a week or ten days, even at the most favorable temperature. After the long incubation period, however, the protein is vigorously attacked with the formation of the usual putrefaction products. When mixed with other organisms, as for example *Staphylococcus aureus* or *Proteus vulgaris*, the preliminary period is relatively short and putrefactive decomposition takes place much sooner. These observations are in accord with those of Sturges and Rettger (1919).

*C. putrificum* has a very marked peptolytic action in ordinary peptone broth, as may be shown readily with the aid of the ammonia, Sörenson, Van Slyke and quantitative biuret tests. The complex nitrogenous substances in the commercial peptone are destroyed rapidly, with the formation of large amounts of ammonia, but with little permanent increase in amino nitrogen. The biuret figures drop sharply (chart 2).

Important points of difference between *C. putrificum* and *C. sporogenes*, *C. tertium* and *C. cochleareus*, organisms with which the Bienstock anaerobe has been confused, are as follows: *C. sporogenes* is actively proteolytic and saccharolytic; *C. tertium* is saccharolytic and peptolytic, but not proteolytic; and *C. cochleareus* (according to the statements of the British Medical Research Committee) is saccharolytic and non-proteolytic. *C. putrificum* occupies an entirely different position in that it is proteolytic and but very slightly or not at all saccharolytic. There are sufficient morphological and cultural differences to set the Bienstock anaerobe apart from the others, and not only should it be easy to distinguish *C. putrificum* from these three organisms, but from all known anaerobes by a combination of the morphological, cultural and biochemical characters, which have been mentioned above.

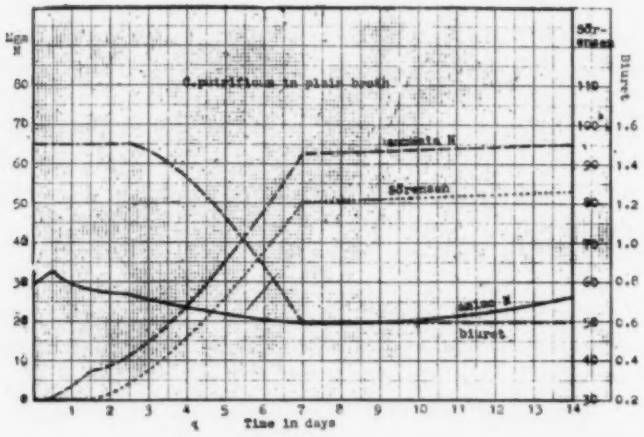


CHART 2. PLOTTINGS OF AMMONIA, SÖRENSEN, AMINO NITROGEN AND BIURET FIGURES FOR *C. putrificum* IN PLAIN BROTH

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# TRANSPARENT MILK AS A BACTERIOLOGICAL MEDIUM

J. HOWARD BROWN AND PAUL E. HOWE

*From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, New Jersey*

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The addition to milk of an oxalate or a citrate, in the form, for example of a sodium salt, will cause the opacity of milk to disappear and give a solution which is opalescent in thick layers but almost clear in thin layers.[1] A similar result may be obtained with sodium sulphate when added in relatively larger amounts. This change is particularly evident when the milk is diluted slightly. Although we have not found the phenomenon described in the literature it may have been observed by those who for infant feeding have added sodium citrate to milk to prevent rennet coagulation. The general nature of the reaction has been known and the procedure has been used to prevent the coagulation of blood. Arthus (1902) showed that sodium citrate in the proportion of 2 to 3 parts per 1000 would prevent the coagulation of milk by rennin. Bosworth and Van Slyke (1914) in a study of "why sodium citrate prevents curdling of milk by rennin" found that there was an increase in the amount of soluble calcium which would pass through a Chamberland filter with increasing quantities of sodium citrate until approximately 1 gram of the hydrated salt had been added to 100 cc. of milk. Rennin coagulation was prevented when 0.4 gram of the citrate had been added to 100 cc. of milk. The failure to coagulate in the presence of citrates was ascribed to the formation of calcium citrate or sodium-calcium citrate.

The loss of opacity is apparently due to the removal of the calcium combined with casein or globulins in milk. The addition

[1] Howe, Paul E., unpublished work.

of oxalate or sulphate results in the formation of insoluble calcium oxalate or sulphate which may be removed by centrifugation. By the addition of sodium citrate, however, the calcium of milk is not precipitated (Arthus, 1902), but remains in solution in a non-effective form in so far as its ability to combine with casein or to react with the para-casein formed as a result of the action of rennin are concerned. We have not found an adequate explanation of the nature of the calcium-citrate combination. That it is in solution is indicated by the work of Arthus and of Bosworth and Van Slyke. We have evidence of this in the fact that during each sterilization there is formed a heavy precipitate of a calcium salt which we assume to be calcium citrate. It has the property of calcium citrate of being precipitated when heated and redissolving upon cooling. On the other hand, the calcium in solution in the clarified milk must be sufficient to form the insoluble para-casein compound for there is enough calcium in milk to do so and in the preparation of transparent citrated milk none need be removed. Sabbatani (1902) states that the effect of the citrate in blood is due to a reduction in the number of calcium *ions* in solution. He also shows that the ratio of citrate to calcium for the prevention of coagulation is three to one. Arthus holds that there is a specific effect of the citrate ion which inhibits flocculation, in addition to any changes which may take place in solubility.

In the preparation of transparent milk as a bacteriological medium we have diluted 1 part of skim milk with 2 parts of distilled water and then added 0.4 per cent of sodium citrate. After standing for about an hour the clarified milk may be filtered through paper though this is hardly necessary if the mixture is allowed to stand sufficiently long. To avoid caramelization of the milk sugar during sterilization the reaction of the medium is adjusted to about pH 6.8. The medium is then tubed and sterilized fractionally. During each steaming in the Arnold sterilizer a heavy precipitate is thrown down but redissolves as the medium cools. There finally results an almost water-clear medium without any precipitate whatever. Oxalated milk may be prepared in the same manner except that the fine precipitate of

calcium oxalate should be removed by centrifugation. For most purposes the citrated milk appears to be the more satisfactory medium, since the citrate gives changes which correspond with those which take place in milk without further treatment. Citrates are a normal constituent of milk.

We have used these media for the cultivation of a number of organisms—streptococci, anaerobes, and members of the colon-typhoid group. A few organisms grow better in the citrated milk than in oxalated milk, though the latter is useful for special purposes because of the removal from it of the calcium. The citrated milk shows all the cultural reactions which may be observed in untreated milk of the same dilution. Some organisms, notably the paratyphoids, produce a reaction not to be observed in untreated milk. These produce in citrated milk a milky translucence or opacity which we attribute to the decomposition of the citrate with liberation of calcium in the ionized state, an appearance which may be produced artificially by the addition of a small amount of calcium chloride to the sterile medium. This reaction is not produced by cultures in oxalated milk from which the calcium has been removed.

The cultural reactions which we have observed in transparent citrated milk may be summarized as follows:

I. Neither acid production nor digestion of casein. Reaction alkaline or neutral
   - *a.* The medium remains clear except for the clouding due to visible growth. The addition of a few drops of $CaCl_2$ solution causes it to become milky. Example: Bact. typhosum. Bact. alkaligenes
   - *b.* The medium becomes milky, probably due to a release of ionized calcium. Example: Bact. paratyphosum, Bact. cholerae-suis

II. Acid production
   - *a.* A small amount of acid may do nothing more than change the color of the indicator which may be added to the medium. Example: Strep. pyogenes
   - *b.* A larger amount of acid may produce translucence. Often observed as a transitory reaction. Example: Bact. cloacae
   - *c.* Large amounts of acid produce coagulation or precipitation of casein. Example: Strep. lacticus, Lactobacillus bulgaricus, Bact. coli, Clostridium welchii

III. Rennin production
   - *a.* Without release of calcium from citrate should give precipitate when calcium chloride is added unless the casein has been digested

b. With release of calcium from citrate should produce precipitate or coagulum

IV. Casein digestion

a. Should give diminished or negative precipitation upon addition of acetic acid. Example: Proteus vulgaris

Various combinations of the above reactions may be observed and there are doubtless other reactions which are not mentioned above, for instance, with alkali production we have sometimes observed a thickening of the medium into a transparent jelly. This reaction was produced by Bact. alkaligenes.

## SUMMARY

Milk may be transformed into a transparent culture medium by the addition of small amounts of various salts. Sodium citrate seems to be the most suitable for this purpose.

In such a medium there may be observed not only the ordinary reactions of various bacteria in milk but also some others not observed in untreated milk.

The principal advantages of the transparent milk as a medium reside in the greater visibility of changes which occur in it. Indicators are much more easily seen in it than in opaque milk. As long as the acidity remains below pH 5.5 colorimetric hydrogen ion determinations are easily made. Clouding and the formation of sediment due to bacterial growth may be observed as in bouillon when no visible change whatever is produced in ordinary opaque milk.

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# THE USE OF AGAR SLANTS IN DETECTING AMMONIA PRODUCTION AND ITS RELATION TO THE REDUCTION OF NITRATES

G. J. HUCKER AND W. A. WALL

*New York Agricultural Experiment Station, Geneva, New York*

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The use of agar slants in detecting the production of acid by bacteria has been reported by our laboratory (Conn and Hucker, 1920), and used successfully for some time. The determination of such physiological activities on solid media has many advantages over the older methods in which liquid cultures were used and especially is this true in the case of many of the free-living organisms which either fail to grow or grow very poorly in liquid media. As ammonia production by bacteria has an important significance in assigning organisms to their natural groups, an effort has been made to adapt some of the many ammonia tests to use with solid media. One of the chief difficulties in employing the usual methods has been the fact that the presence of certain organic compounds interferes with the reaction and, as all cultural media contain more or less organic material, satisfactory results could not be obtained. This is especially true where Nessler's reagent is used as this has a special affinity for organic nitrogenous material especially the aldehydes. Because of this fact, it has been impossible to use glucose as a source of carbon in a medium in which bacteria are grown to be tested for ammonia production.

Bacteria in their metabolism are usually considered as securing their energy from the oxidation of various carbon compounds, while the nitrogen required to produce the bacterial protoplasm is derived from various organic sources and in some cases from inorganic compounds. The utilization of protein and other organic sources of nitrogen, whether for energy or for protoplasm building, is in many cases accompanied by free ammonia pro-

duction. Consequently, the presence or absence of this particular by-product of bacterial metabolism indicates fundamental activities of the bacterial protoplasm.

In the determination of ammonia production the Thomas (1912) test, which has been used in part of Ayers, Rupp and Mudge (1921), in liquid cultures for studying certain strains of streptococci, has been found to be exceedingly helpful in our laboratory either in the presence or absence of organic matter.

The organisms to be tested are grown on the following medium:

| | |
|---|---|
| Peptone | 4.0 per cent |
| Glucose | 0.2 per cent |
| Dipotassium phosphate | 0.5 per cent |
| Agar | 15.0 grams |
| Water | 1000.0 cc. |

The culture is incubated at the optimum temperature, preferably for one week, and 1 cc. of each of the following reagents added to the surface of the slant: 1 per cent phenol solution and sodium hypochlorite (1 per cent available chlorine). The tubes are allowed to stand for one-half hour and if ammonia is present, a decided blue color appears.

As an optional method, an adaptation of the common Sorensen method has also proved satisfactory for use with agar slants which contain no peptone or other organic nitrogen. As a reagent, 2 cc. of neutral formaldehyde (containing a few drops of phenolphthalein) are added to each slant. Ammonia production is indicated by the presence of acid which decolorizes the added reagent. The absence of ammonia is indicated by no change in the reaction of the fluid on the surface of the slant.

Neither of these tests are new; but their adaptation for use with solid media with free-living organisms, many of which fail to grow in liquid media, has been helpful in studying their physiological activities.

These tests are also of special importance in connection with the determination of nitrate reduction, for in some cases a negative nitrite reaction in a nitrate medium does not indicate failure to reduce nitrate. Some organisms may convert the nitrite as rapidly as it is formed either into ammonia or into the protein

of their own bodies. In the former case an ammonia determination may supply the missing information. The organisms to be thus tested may be grown either on a synthetic medium containing no nitrogen except nitrate, or if they fail to grow under such conditions on a nitrate-peptone medium. In the latter case a duplicate inoculation must be made on to a similar peptone medium without nitrate to be sure that the ammonia is not produced from the peptone. For a synthetic medium the writers recommend that described by the Committee on the Descriptive Chart (1920) except that glucose be substituted for sucrose as it is a more available sugar than the latter and its presence is not confusing when the Thomas test instead of the Nessler test is used to detect ammonia. This medium is:

| | |
|---|---|
| Water | 1.0 liter |
| Agar | 15.0 grams |
| Glucose | 10.0 grams |
| Potassium nitrate (other inorganic nitrogen sources may be used if desired | 1.0 gram |
| Calcium chloride | 0.5 gram |
| Dipotassium phosphate | 0.5 gram |

If these tests are used in routine work for those organisms that do not show nitrite as ordinarily tested in nitrate medium, much valuable information can be obtained. It has been found in this laboratory, for instance, that out of several hundred cultures examined about 60 were nitrite-negative when grown on nitrate-peptone agar. Of these 60 it was found, however, that 4 produced ammmonia on the above synthetic media without any evidence of nitrite formation. By the usual methods such organisms would be classed as non-reducers, whereas in the absence of all other possible sources of ammonia, the positive ammonia-reaction plainly indicates that they must have reduced the nitrates to ammonia, and in such cases to class them as non-reducers would be a decided error.

As a routine procedure in connection with nitrate reduction such a combination of tests shows promise of giving interesting data on the physiological activities of bacteria.

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# METHODS OF PURE CULTURE STUDY

## REPORT OF COMMITTEE ON BACTERIOLOGICAL TECHNIC

H. J. Conn, *chairman*, K. N. Atkins, H. J. Brown, F. Eberson, G. E. Harmon, G. J. Hucker, F. W. Tanner, and S. A. Waksman

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Several years ago the committee on the descriptive chart was asked by the Society to prepare a manual of methods to be used in connection with the chart, in the pure culture study of bacteria. As at that time the methods were felt to be very crude, and as considerable work was then in progress on them, it seemed that the best thing that could be done was merely to draw up a short report on the subject, which was printed in the Journal (Committee on Descriptive Chart, 1917) and was distributed in separate form with the charts that were ordered. A limited edition of these separates was printed with the expectation of getting out a revision shortly. Such a revision was prepared a few years later (1920) and at the present time there is need of a second revision.

This work has now been put in the hands of the more recently organized committee on bacteriological technic and this committee has drawn up at the present time a third edition of the report on methods of pure culture study which is to replace the second edition now almost out of print. So much material has been collected, however, in the last few years and the methods are now so much more satisfactory than they were when the first edition was prepared that it seems advisable to the committee to make this third edition a separately published manual of methods, according to the instructions given to the earlier committee.

This manual, as now prepared, contains to a large extent methods that have been printed in the earlier editions of the report; but for certain procedures, new methods have been described.

These new methods are now published to call them to the attention of the Society for discussion and criticism before the manual is printed. At the next meeting of the Society the committee expects to ask for approval in publishing this manual of methods.

When asking approval to print this manual, the committee is not to ask that the methods included therein be made official. The committee has consistently taken a stand against official methods for research work and does not wish that these methods be construed as such. For this reason the committee does not want the methods officially adopted by the Society, but merely wants approval for the plan of publishing them in a manual of methods recommended for pure culture study.

The following pages give the new methods that are included in this report.

## SPECIAL STAINS

Of these the Gram stain has been given particular attention by the committee. In the last edition of the report on methods (1920) the Stirling modification was recommended; but in recent tests it has proved to be less satisfactory than certain other anilin oil methods. In the first place it has been found to have no special advantages as to keeping quality; and in the second place it gives preparations showing a large amount of precipitate; and in the third place a careful search through the literature has so far failed to show the original place of publication of this technic;[1] and finally, the methods employed by various bacteriologists under this name differ very greatly from one another. At present it seems best to recommend three different procedures. The first calls for Ehrlich's anilin gentian violet with the technic given in Buchanan's Veterinary Bacteriology (p. 103) and is the anilin oil formula which has given the best results in recent tests. The second method is the ammonium oxalate

[1] The committee will be very grateful to any one who can furnish information as to where this Sterling technic was originally published, if it was actually published, and if not, where it was originally described.

method (Hucker, 1922) and the third that of Atkins (1920), both of which are highly to be recommended on account of the keeping quality of the solutions, but have not yet been compared with the older methods sufficiently so that the committee can be sure that they can replace them.

*Method 1*

Ehrlich's anilin gentian violet[2]

| | |
|---|---|
| Gentian violet (saturated alcoholic solution) | 6 cc. |
| Alcohol | 5 cc. |
| Anilin water (98 cc. water to 2 cc. anilin oil) | 50 cc. |

Lugol's iodine solution

| | |
|---|---|
| Iodine | 1 gram |
| Potassium iodide | 2 grams |
| Water | 300 cc. |

*Method 2 (Hucker's)*

| | |
|---|---|
| Gentian violet (saturated alcoholic solution) | 1 part |
| Ammonium oxalate (1 per cent aqueous solution) | 4 parts |

Lugol's iodine solution (as usual)

*Method 3 (Atkins')*

| | |
|---|---|
| Gentian violet (saturated alcoholic solution) | 1 part |
| Anilin sulphate (1 per cent aqueous solution) | 3 parts |

Atkins' iodine solution

| | |
|---|---|
| Iodine | 2 grams |
| Normal NaOH | 10 cc. |
| Water | 90 cc. |

Technic. Stain one minute; treat in iodine solution one minute; decolorize one minute with methods 1 and 2, five minutes with method 3; counterstain about ten seconds. For the counterstain use 1:10 safranin (i.e., 1 part saturated alcohol solution to 10 parts water) 1:10 eosine or 1:10 bismark brown or pyronin. With method 1 do not wash between the different procedures; merely drain thoroughly with the other two methods wash between each step.

[2] The committee has found crystal violet to be a very satisfactory substitute for gentian violet in all these formulae. It is of much more constant composition, and therefore to be preferred to the very variable gentian violet. See the following paper, p. 533.

FERMENTATION OF SUGARS, ALCOHOL AND GLUCOSIDES

This may be determined in either solid or liquid media according to which the organisms to be investigated prefer for their best growth; but the committee recommends that solid media be used whenever possible.

The method recommended for determining fermentation in solid media is that described by Conn and Hucker (1920).

When solid media are to be inoculated, use the following agar slant method: Prepare two lots of agar media, one the regular beef extract agar and the second a peptone-free agar having the following composition:

| | | |
|---|---|---|
| $NH_4H_2PO_4$ | 1.0 gram | Adjusted to pH 7 by the addition |
| KCl | 0.2 gram | of NaOH. About 6 cc. normal |
| Agar | 15.0 grams | NaOH required |
| Water | 1000 cc. | |

To both of these media add 1 per cent of the fermentable substance to be investigated and 2 cc. per liter of a saturated aqueous solution of brom cresol purple. Distribute these two media in test tubes and cool in a slanting position. The tubes should be inoculated either on the surface alone like an ordinary agar slant, or partly on the surface and partly in a stab at the base of the tube. The tubes should be inoculated at 37°C. or 25°C. according to the optimum temperature of the organism under investigation. Examine after twenty-four hours and then as often as seems necessary according to the rapidity of the growth so as not to overlook any changes in reaction. Acid can be readily determined by the yellowing of the indicator. A very good idea as to the amount of acid can be obtained by noticing the size of this zone of yellow. This method is only roughly quantitative yet gives fairly satisfactory results. A convenient method of denoting results is the following: use a single + sign if acid is produced but the yellow zone does not extend to the base of the slant, denote ++ if the acid zone extends to the base of the slant but no further, +++ if it extends half way from the base of the slant to the bottom of the tube and ++++ if the whole tube of agar has turned to its acid color. Use the symbol (0) to indicate no change in reaction.

Interpreting results the two media should be taken into account as some organisms produce more easily detectable acid on the peptone media, some on the peptone free media. In the former case it may be assumed that the acid produced by the organisms is obscured either by the buffer of the peptone or by the alkalinity produced from it.

If it is decided to observe the production of alkalinity as well as acidity, it is recommended that two indicators be used, e.g., brom cresol purple with cresol red (see 1920 report, p. 130). The use of these two indicators is decidedly recommended because in many cases it is instructive to observe the production of alkalinity. Some organisms produce acid in one part of the media and alkali in another; when this occurs it is to be noted. Denote alkali with a − sign.

In using solid media, the production of gas can be quite readily detected by the presence of bubbles and cracks in the agar. It would seem theoretically that this is a less accurate way of determining gas production than the fermentation tube; but wherever the two methods have been compared in the case of organisms growing well or better on solid media, agar slants have been found to give as reliable results as fermentation tubes.

When liquid media are to be inoculated, prepare two media similar in composition to the above mentioned but without agar, sterilize in fermentation tubes and incubate at 37° or 25° according to the optimum temperature of the organism in question. The media may contain the same indicator or indicators as those already mentioned in the case of solid media, in which case the cultures should be examined at intervals, as often as seems necessary to record changes in the reaction. Although there is ordinarily no appreciable influence of the indicator on the growth of the organism still there may be cases when the investigator prefers using media without an indicator; in this case the culture should be tested ordinarily on the first, third and seventh days, although the best days of testing will depend on the rapidity of growth of the organism. To test for acid, use an indicator having a range which covers the reaction of the culture. If possible compare the culture with a standard buffer solution.

REDUCTION OF NITRATES

The following procedure is recommended: Inoculate first into nitrate broth and on to slants of nitrate agar (containing 0.1 per cent $KNO_3$ plus beef extract and peptone as usual). Test the cultures on various days as indicated on the chart. On these days examine first for gas as shown by foam in the broth or by cracks in the agar. Then test for nitrate with the following reagents.

1. Dissolve 8 grams sulphanilic acid in 1 liter of 5 N acetic acid (1 part glacial acetic acid to 2.5 parts of water), or in 1 liter of dilute sulphuric acid (1 part concentrated acid to 20 parts water).

2. Dissolve 5 grams $\alpha$-naphthylamine in 1 liter of 5 N acetic acid or of very dilute sulphuric acid (1 part concentrated acid to 125 parts water).

Put a few drops of each of these reagents in each broth culture to be tested, and on the surface of each agar slant. A distinct pink or red in the broth or agar indicates the presence of nitrite. It is well to test a sterile check which has been kept under the same condition to guard against errors due to absorption of nitrite from the air. Presence of nitrite or of gas shows the nitrate to have been reduced. A negative result does not prove that the organism is unable to reduce nitrates; in such a case further study is necessary as follows:

In case the fault seems to lie in poor growth, search should be made for a nitrate medium in which the organism in question does make good growth by means of the following modifications: increasing or decreasing the amount of peptone; altering the reaction; adding some readily available carbohydrate. Presence of nitrite in any nitrate medium whatever should be recorded as nitrate-reduction. Presence of gas should be similarly interpreted, provided there is no other substance (e.g., sugar) present in the medium, from which the organism under investigation is able to produce gas.

In the case of those organisms which are nitrite-negative and produce no gas in nitrate-peptone agar or broth, but which show

good growth in one or both media, proceed as follows before concluding that the organisms do not reduce nitrate:

Inoculate into:

A. Nitrate-peptone agar or broth.

B. Peptone agar or broth without nitrate.

C. Synthetic nitrate medium (formula recommended: Nitrate 1 g, $K_2HPO_4$ 0.5 g, $CaCl_2$ 0.5 g, glucose 10 g, distilled water 1000 cc., with or without agar according to the organism under investigation).

D. Peptone agar with 2 p.p.m. potassium nitrite.

Prepare the following reagents for the Thomas test for ammonia:

1. A 5 per cent solution of phenol,

2. A solution of sodium hypochlorite containing one per cent available chlorine. To obtain this amount of available chlorine, the solution should be so adjusted that 1 cc. of it should neutralize 2.86 cc. of a N/10 solution of sodium thiosulphate (i.e., 24.8 grams to the liter), titrating with starch as an indicator in the presence of acetic acid and potassium iodide.

In making the Thomas test 1 cc. of each of the above reagents should be added to the broth or agar slant[3] on which the culture has been growing and allowed to stand half an hour. A blue color indicates the presence of ammonia.

After incubation, test A and B for ammonia by the Thomas method; test C for nitrite as usual, and for ammonia by the Thomas method; test D for nitrite. The results give the following indications:

On A: Presence of ammonia indicates nitrate reduction if no ammonia is present in B. Absence of ammonia on both media indicates non-reduction provided this conclusion is confirmed by the results on C and D. Otherwise inconclusive.

On C: Presence of either nitrite or ammonia indicates nitrate reduction. Absence of both, if the growth is good, strongly indicates non-reduction.

On D: Presence of nitrite indicates non-reduction in case this is confirmed by the two above tests, for if the organism cannot destroy

[3] This test can be made for this purpose to great advantage with agar slant culture as well as with liquid media, as recently shown by Hucker and Wall (1922).

the extremely small amount of nitrite present in D it is not likely to have destroyed the nitrite in nitrate agar as fast as it produced it.

The organisms for which inconclusive results must be recorded are those which produce ammonia in B, which fail to grow on C, and which destroy the nitrite in D. The number of organisms in this group is probably very small.

### INDOL PRODUCTION

The indol test has always been one of the most unsatisfactory of those that have been used for characterizing bacteria. This has been true, partly because of the variable composition of the media used, and partly because of the inaccuracy of the tests used for detecting the presence of indol.

The first of these objections (i.e., the variable composition of the media) Zipfel (1912) has tried to avoid by using tryptophane in place of peptone. As this medium is not satisfactory for routine use other investigators have tried to accomplish the same purpose by using a solution of peptone treated with trypsin. Frieber (1921) for instance used a medium of this kind which he prepares as follows:

To 1 liter of ordinary peptone bouillon he adds 0.2 gram of trypsin, then adds chloroform and toluol to prevent bacterial growth and incubates for twenty-four hours to forty-eight hours at 37°, subsequently filtering and diluting with three parts of physiological salt solution. In the absence of conclusive data as to the advantages of this medium, the committee recommends that organisms be tested both in ordinary peptone solution and in the trypsinized bouillon of Frieber.

The second objection to indol tests, namely, the inaccuracy of methods for detecting indol has been discussed in some length by Frieber. Indol has generally been detected by the Salkowski method, that is through the use of sodium nitrite and sulphuric acid. This test has been realized for some time to be an inaccurate one and recently Frieber has shown quite conclusively that this reaction is positive not only with indol and with the methyl-indols but with indol-acetic-acid as well. The most satisfactory test for indol is now regarded to be Ehrlich's test which gives a positive reaction only with indol itself and with

α-methyl-indol. The Ehrlich reagent, however (p-dimethyl-aminobenzaldehyde), is rather too expensive to use in routine laboratory work. For this reason tests with other reagents are often preferred, as for example the Vanilin test. The Vanilin test is a very convenient one to make and gives a more nearly true indol reaction than does the Salkowski test. Frieber shows, however, that it gives a positive reaction not only with the compounds that give the Ehrlich test but also with β-methyl-indol.

In the light of this contribution to the subject the committee suggests the following procedure, pending further investigation of the method: Test all the cultures by the Salkowski reagent: then test the positive cultures further with the Vanilin test; and lastly test those that are positive to Vanilin with the Ehrlich reagent. Record on the chart what reaction is obtained with each test used, because it is very evident that the different reagents indicate the presence of different compounds.

The Salkowski test is made as follows: Mix 5 cc. of the culture with about one-third its volume of 1:1 sulphuric acid. Then add on the surface a small amount of a 0.02 per cent solution of sodium nitrite. A positive reaction is indicated by a pink zone between the acidified culture fluid and the nitrite solution.

The Vanilin test is as follows: To 5 cc. of the culture add 5 drops of a 5 per cent solution of vanilin in 95 per cent alcohol and 2 cc. of concentrated sulfuric acid. Indol gives a clear orange by this test which reaches its greatest depth in two or three minutes. Tryptophane, on the other hand, gives a reddish violet which develops more slowly and deepens on standing or heating.

The Ehrlich test is made as follows: The reagent is a 2 per cent solution of para-dimethylaminobenzaldehyde in 95 per cent alcohol. One cubic centimeter of this reagent is added to the culture, then drop by drop concentrated hydrochloric acid is added until a red zone appears between the alcohol and peptone solution. Not more than 0.5 cc. of the acid is required. On standing the zone deepens and widens. The red color is soluble in chloroform and the test may be confirmed by shaking the culture with chloroform to see if the pigment dissolves If it proves soluble the test is considered positive.

### PRODUCTION OF HYDROGEN SULFIDE

The method of determining hydrogen sulfide which seems to be in most common use is the lead acetate agar method. This

method was described by Kligler (1917) and has been reported upon favorably from other sources. Although certain weaknesses in the technic have been pointed out it is given here as a provisional method. The committee has not yet had an opportunity to give it the investigation it needs and therefore does not indorse the method.

Prepare an agar like the standard peptone-beef-extract formula but with 30 grams instead of 5 grams of peptone per liter, and with a reaction between pH = 7.2 and pH = 7.6 (i.e., just at the alkaline, or blue end of the sensitive range of brom thymol blue). Sterilize this medium 5 cc. to the tube. Sterilize separately a 0.1 per cent solution of basic lead acetate, and after sterilization add 5 cc. to each tube of agar, when the agar has cooled to about 50°C. Allow the tubes to cool in an upright position, and incubate for a long enough period to be sure that no contamination is present. Inoculate these tubes with the organisms to be tested by stabbing with an inoculating needle. Incubate at optimum temperature for from eighteen hours to a few days according to the vigor of the culture. Hydrogen sulfide causes a blackening or browning of the medium along the line of inoculation.

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# AN INVESTIGATION OF AMERICAN GENTIAN VIOLETS

## REPORT OF COMMITTEE ON BACTERIOLOGICAL TECHNIC

PREPARED BY H. J. CONN, CHAIRMAN

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This report is a continuation of the work on American stains that has recently been published in this journal (Conn, 1922). In this earlier report fairly definite conclusions were reached in regard to fuchsin and methylen blue but the work on gentian violet was regarded as being unfinished.

Gentian violet is a term which does not refer to any definite chemical compound, but rather to a mixture of dyes of a certain group. The term was apparently originated by Grübler and is not recognized in the dye industry or in the textile industry. The dyes in the mixtures sold as gentian violet all belong to the pararosanilin series but at the present time the different manufacturers and dealers nearly all sell different mixtures of these dyes under the name of gentian violet. This being the situation, the best use for the term gentian violet seems to be as a generic designation for any violet pararosanilin which has the general staining properties recognized as belonging to Grübler's gentian violet.

As mentioned in the earlier report the pararosanilin base has the formula

$$H_2N \cdot C_6H_4 \cdot C(OH) \begin{matrix} C_6H_4 \cdot NH_2 \\ C_6H_4 \cdot NH_2 \end{matrix}$$

The six hydrogen atoms of this formula which are in the amino-groups can be replaced by a methyl group, an ethyl group or a benzyl group. The most commonly known compounds of the pararosanilin series contain in these positions 4, 5, and 6 methyl groups respectively, and are known as the tetramethyl, penta-

methyl and hexamethyl pararosanilins. The mixtures of lower methylation have the most reddish cast of all the dyes in the series and are generally designated as methyl violet. As the mixture contains larger and larger amounts of more highly methylated compounds, it becomes bluer in color and the successive deeper shades are known in the textile trade as methyl violet B, methyl violet 2B, methyl violet 3B, etc. Those compounds designated as methyl violet 5B, 6B, and 7B, respectively, are still bluer in shade and apparently contain a pararosanilin in which a benzyl group has been introduced into one of the amino-groups.

The only textile dye in this series which if pure has a definite chemical formula is crystal violet, which is hexamethyl pararosanilin. This dye is quite a deep blue violet.

In the earlier work already reported a comparison was made of various commercial samples of methyl violet, methyl violet B, methyl violet 2B, methyl violet 6B and crystal violet, which were compared with certain samples obtained from different biological supply houses labeled as gentian violet. It was found that crystal violet and methyl violet 6B were at least fair substitutes for gentian violet in the Gram stain but that the methyl violets of lower methylation were unsatisfactory for this purpose. The present investigation was planned as a more intensive study of crystal violet and methyl violet 6B in comparison with gentian violet.

In the second series of tests there were included 10 samples of crystal violet, 8 samples of methyl violet 6B, and 10 samples of gentian violet. Of these 28 samples, 19 had been included in the earlier work.

Two of the samples of crystal violet included in the earlier work, namely those from the Providence Chemical Company, and from Dicks David and Company, unfortunately had to be omitted from the second test, due to the exhaustion of the samples that had been investigated; but on the other hand in the present work three samples of crystal violet, four of methyl violet 6B, and two of gentian violet were added which were not included in the first tests. Of these 9 additional samples, three were

Grübler's, but they were distributed to the collaborators designated by number only, and were in most cases thought to be American samples.

The collaborators were instructed to use these samples of stain in staining bacteria with the Gram technic. To make the results more constant if possible than in the earlier work, directions for making the tests were furnished and cultures were distributed for use as test organisms. The cultures furnished were: Bacillus cereus, as a strongly gram-positive organism; a small short rod of the fluorescent group, as a decidedly gram-negative organism; and lastly an unidentified micrococcus which had been found to be weakly Gram-positive although often negative with poor technic. The directions for making the stains which were furnished were as follows:

Twenty-four hour cultures should be used in the test, the coccus incubated at 37°, the other two at room temperature (or 25° if available).

To make results strictly comparable the procedure should be carefully controlled and timed as follows:

Stain thirty seconds.
Drain but do not wash.
Iodine thirty seconds.
Drain but do not wash.
Ninety-five per cent alcohol until no more stain is removed; not over two minutes.
Keep in agitation, and transfer without washing to
Counterstain thirty seconds.
Wash and dry.

*Stain formulae*

The gentian, crystal or methyl violet may be made up according to one or both of the following formulae. If only one formula is used, select formula A; but if convenient it is hoped that formula B may also be used for the purpose of comparison.

| *A. Regular formula* | cc. | *B. Optional formula* | cc. |
|---|---|---|---|
| Saturated alcohol solution of stain | 6 | Saturated alcohol solution of stain | 10 |
| 95 per cent alcohol | 5 | 1 per cent ammonium oxalate solution | 40 |
| Anilin water (1:49, filtered) | 50 | | |

BOOK B

TIGHT

BOUND

HTLY

Lugol's iodine solution should be used (1 iodine: 2 KI : 300 water.)
The counter-stain may be selected according to personal preference.

Basic fuchsin ..... (1:10)
Safranin ..... (1:10)
Eosin ..... (saturated alcohol)

Bismark brown (2 per cent) may be used, but the staining period should be longer.
In making reports be sure to specify the counter-stain used.

Of these two formulae the one containing anilin (A) was selected because in a series of preliminary tests made with 4 different samples of gentian violet and using 20 different technics, this particular formula was among the best if not the very best. The ammonium oxalate formula (B), however, proved almost if not quite as good in these preliminary tests and the solution keeps indefinitely. It was such a new formula, however, that it did not seem best to include it in the present series of tests as a regular formula; hence formula (A) was selected for this purpose with the ammonium oxalate method as an optional formula, in order to get some information as to how well the latter compared with the anilin formula. Five investigators, it will be seen, used formula B and obtained as good results with it as with formula A.

The results of the tests are given in table 1. In this table the same method of indicating results is used as was employed in the earlier article. The significance of the symbols used is as follows:

E, excellent
G, good
F, fair
U, unsatisfactory

One column of this table indicates the average rank of the first tests, that is those reported in the previous paper, and another column indicates the average rank in the present series of tests.

The first thing to be noticed in the present series of tests is that all the samples have nearly the same rank in their average grade, which is generally G, G + or G −. Provided all the results

*Results of second series of tests of gentian violet (etc.), samples for Gram stain*

| Formula | MACY | | STOVALL | | HUNTOON | | HOOS | HEDGWITZ | MCCONNELL | FLEISHER | HARRISON | | BACKETT | HITTEN | | HOCHSTEL | WILLIAMS | HEALY | | FELLNER | HAMMON | HUNTER | GORHAM | AVERAGE GRADE FIRST TESTS |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | A | B | A | B | A | A | A | A | A | A | A | B | A | A | B | A | A | A | B | A | A | A | A | |
| *Crystal violet* | | | | | | | | | | | | | | | | | | | | | | | | |
| Du Pont | F | G | E | G | | | | | | G | | | | | | G | G | E | E | G | F | E | | G+ G− |
| Eimer and Amend | F | G | | | | | | | | | E | E | E | U | U | | | | | | F | E | | G− G− |
| Harmer | | | | | | | | | F | | | | U | E | G | | | F | U | | F | E | | F+ G− |
| Heyl | | | | | | | | | E | | E | E | E | U | U | | | U | | | F | | | G− G− |
| Coleman and Bell | F | G | | | E | F | F | | | F | G | | | | | E | E | | | G | F | | | F G |
| Goldin | | | E | G | E | | F | F | | G | E | E | | | | E | U | | | U | F | | | G− G− |
| H. S. Labs | F | G | | | | | | | | G | E | E | | | | | | E | E | | F | | | F+ G |
| Phys. Lab. Supply | | | | | | | | | E | | G | | E | U | U | | | E | E | | F | | | G F+ |
| Leitz | | | E | F | F | F | G | F | | | | | | | | E | U | | | G | | | | F+ G− |
| Grübler | | | U | U | U | | F | F | | | | | | | | E | G | | | | | | | G− G− |
| *Methyl violet 6B* | | | | | | | | | | | | | | | | | | | | | | | | |
| Newport | | | | | G | G | F | G | E | | U | E | E | U | F | | | E | E | | | | | | G− G− |
| Providence | | | E | U | G | U | F | G | | G | E | U | | G | | | | G | F | | | | | | G− G− |
| Heyl | G | G | | | U | G | E | E | E | | G | | E | F | E | | | | | | | | | | G− G− |
| Coleman and Bell | | | E | G | U | G | U | G | E | G | E | E | | | | | | E | E | | | | | | G− G− |
| Goldin | F | F | | | G | G | G | F | E | G | E | E | G | | U | F | E | | | | | | | | G− G− |
| H. S. Labs | G | G | | | | | | | | G | | | G | G | | | E | | | | | | | | E− G |
| Leitz | | | E | U | F | F | F | U | G | | G | G | | | | E | F | F | G | F | F | E | | | G+ F+ |
| Grübler | E | E | U | F | F | U | G | F | E | E | G | G | | | | | | F | F | G | E | | | | F+ G− |
| *Gentian violet* | | | | | | | | | | | | | | | | | | | | | | | | |
| Eimer and Amend | E | E | U | F | G | G | F | G | G | G | F | | | | | G | G | G | F | | G | | | | F F |
| Harmer | | | | | | | | | | | | F | E | E | G | E | F | F | G | F | E | | | | G+ F+ |
| Providence | E | E | F | F | U | G | F | | E | E | G | | | | | | | E | G | | | | | | F+ G+ |
| Heyl | | | | | G | U | G | | | | | | | | | G | G | G | E | | | | | | G− G− |
| Coleman and Bell | | | E | E | G | G | G | | | E | G | | | | | G | U | E | F | | | | | | G+ G |
| Goldine | G | E | | | | | | | | | | | | | | | | | | E | E | E | | | E− F |
| H. S. Lab | | | | | | | | | G | | E | U | E | G | E | | | | | | G | F | | | G G |
| Phys. Lab. Supply | | | | | | | | | | | E | E | E | U | F | | | | | G | E | U | | | G− G |
| Leitz | | | | | | | | | E | G | | E | E | F | F. E | | | | | G | G | F | G | | G F |
| Grübler | | | | | | | | | E | | E | E | F | E | | | | | | | | | G | | G+ G |

are averaged, there is very little choice between crystal violet, methyl violet 6B, and gentian violet. A few more F grades appear with crystal violet than with either of the others but not enough to be of any significance. For our practical purposes these results indicate that the Gram stain can be made with a dye bearing any one of these three names, provided it comes from a satisfactory source.

Quite in contrast to the uniformity of these average results is the variation in the reports from the individual investigators. In spite of the standardized technic used in the work there is not a single sample in regard to which entirely consistent results have been made and practically every sample that has been tested more than four or five times has been reported good or excellent by some investigator and unsatisfactory by some other. This suggests that either the personal equation is one that cannot be eliminated entirely or else that some point in the technic requires further standardization. The chief points which have been mentioned so far as susceptible to further standardization are: temperature at which the cultures are incubated, strength of alcohol used for decolorization, length of time of decolorization, and nature of the counter-stain used. It is very doubtful whether variation could be avoided by controlling all these factors but the committee hopes to plan a further investigation along this line simply to test out some of these points in regard to the Gram technic. In a recent paper Burke (1922) has suggested the importance of some of these factors, but it seems as though a coöperative experiment might give more light on the subject than any study made by a single observer.

In looking up the results of the individual manufacturers of these stains, it is to be noticed first that nearly every sample which has ranked as high as G+ or E− is one that has been tested so few times in this work that the results are not especially significant. For this reason it is regarded as impossible to decide which is the best of the samples tested. It is perhaps of some significance, however, to notice those few samples that have ranked below G− in the general average. These samples are crystal violet of Harmer, H. S. Laboratories, Leitz and

Grübler, the methyl violet 6B of Providence, and the gentian violet of Harmer. With the exception of these few samples there seems no question but that the rest are entirely satisfactory for the Gram stain, and the American samples have ranked practically as well as the Grübler samples. In fact, the Grübler crystal violet ranks the lowest of any of the samples tested.

The chief conclusion from this work seems to be that samples of these three dyes are now produced in this country which are perfectly satisfactory for the Gram stain, apparently just as good as the Grübler samples, and that either crystal violet or methyl violet 6B can be substituted for gentian violet in the gram stain. The committee especially recommends the use of crystal violet for this purpose as it is the one dye of this series for which a definite chemical formula can be indicated, although commercial samples of it are not necessarily pure and may vary in their composition. This variation is probably less than in the case of methyl violet 6B, where the name denotes merely a certain shade of violet, and in the case of gentian violet, where the name is not used in the dye industry and is not mentioned in Schultz's index of dyes. For this reason the committee recommends that those ordering stains to be used in the Gram technic should specify crystal violet instead of gentian violet.

Turning to the results with the different samples of crystal violet, it will be seen that only one of those investigated was from a basic manufacturer, namely the Du Pont sample. This sample and the Goldin sample have given the highest average grade of any of the crystal violets tested. All of the other crystal violets seem to fall slightly below the Du Pont sample which is rather surprising because there are indications to suggest that nearly all of these dealers in biological stains merely rebottle the Du Pont crystal violet and sell it under their own name. If this is the case, the slightly higher rank of the Du Pont sample may be considered to be pure accident.

The only other actual manufacturer of crystal violet in this country of which we have knowledge is the National Anilin and Chemical Company. Their product was not included in

this work but there is reason to believe that it is carefully prepared and cursory examination of slides stained with it by the gram technic indicates that it is as satisfactory as the Du Pont product. In this connection it should be remarked that the Du Pont Company does not sell crystal violet except in bulk, whereas the National Anilin Company is now making a speciality of biological stains and sells them in small containers. If the Du Pont product is desired, it should be ordered through some supply house that can be trusted to supply the actual products specified.

## REFERENCES

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